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WASHINGTON 25, D. C.

LOBUND - ONR

Semi-Annual Progress Report
Contract N6-ori-83, Task Order III

1 January 1952 - 30 June 1952

TO: Chief of Naval Research
Office of Naval Research
Department of the Navy
Washington 25, D. C.

ATTN: Microbiology Branch, Code 443

SUBJECT: Enclosed semi-annual progress report (1 January 1952 to 31 July 1952). Contract N6-ori-83, Task Order III, NR:131-067

DATE: 1 August 1952

I herewith transmit the LOBUND Institute ONR semi-annual report covering the period of 1 January 1952 to 31 July 1952.

There has been no let-up but rather a steady increase in the demands on basic LOBUND Institute facilities from scientists, universities, industries, and research institutes. As pointed out in an earlier report, the justification for Navy participation in the LOBUND research program is further supported by these demands. While the LOBUND-ONR program develops steadily toward standardization of the Reyniers techniques, this program has by no means been completed. We have continued to route the different government agencies and others exhibiting an interest in the LOBUND programs through ONR wherever possible.

The university, recognizing the problem, has authorized the architectural firm of Holabird, Root, and Burgee of Chicago, to make preliminary sketches of facilities necessary to produce a working plant for the production of germ-free animals and the use of these animals at LOBUND Institute by other agencies. All of our experience to date has indicated the great difficulties in trying to ship germ-free animals alive. Moreover, from experience it has become evident to us that it is inadvisable to attempt half-way measures in trying to set up a series of germ-free laboratories elsewhere. It, therefore, seems advisable to strengthen this center and to afford facilities to others until the center is firmly established and all of the many working details of a large scale operation have been satisfactorily completed. If this is done, then one or two other installations could be set up in the future. Germ-free life research, if it is to be used in experimental work, should be operated from a few centers in much the same manner as Oak Ridge or Argonne.

There has been a steady increase of scientific and professional visitors to LOBUND Institute. Many of these are from foreign countries which indicates the growing importance of the institute and the work internationally. Visitors from all portions of the United States also constitute a major load. These visitors are, for the most part, serious

scientists who come to Notre Dame with their problems and the hope that we can find facilities for them to use the Reyniers techniques.

Since the accident on 13 November 1951, which wiped out most of the germ-free colony, we have made a steady comeback against great difficulties and the present colony numbers more than 120 rats. We have in the interim attempted to establish the proper alarms and controls necessary to maintain the production and use of germ-free animals.

We are badly in need of a strictly controlled colony building in which we can maintain disease-free stocks of mammals and birds. We have been quite successful in working out this technique by using a converted quonset hut. However, the colony became contaminated after 18 months operation through the entrance of a wild mouse. The quonset hut after repair has had to be used to house our control experimental animals. Thus we are without, at present, a disease-free breeding stock. It is evident, both from the literature and from the many requests we have had, that there is a great need for a disease-free source of experimental animals. This is especially indicated not only by scientific laboratories, wishing to set up their own stock colonies, but by farmers and agricultural centers into which one or another diseases have crept. We take our mammals by Cesarian section, rear them through weaning germ-free and then carefully contaminate them with the organisms we desire after which they are moved into control animal buildings. Experience has shown that we can operate and keep out of these stocks all pathogens, protozoa, exoparasites, etc. Thus it is possible for us to obtain and supply swine, chickens, and other laboratory animals free from infectious diseases. I believe, based on our successful experience in this area, that we should attempt to establish sufficient facilities to maintain needed stocks and from which we would then be in position to supply any other organizations with the nucleus for developing a clean colony.

We have completed the preliminary work on plastic diving suits for use in situations involving highly infectious microorganisms by other services. These models have been delivered to the government agency needing them.

In this period we have held a colloquium on the growth effects of antibiotics in germ-free chickens. This colloquium was called together to report the preliminary studies made at LOBUND Institute over the past two years. Interest in this problem is very great and it was necessary to know whether or not germ-free chickens would respond by increased growth to antibiotics. The answer is that they do not. The colloquium was very well attended and continues to bring in daily requests for further information and for elaborating these preliminary studies. The colloquium again indicated the very good interest of the scientific body in germ-free techniques and served as an excellent example of the use of these techniques in a very practical problem.

Special attention should be directed to the newly activated program involving germ-free colony apparatus. This is an attempt to simplify the present apparatus and to work out many details of sterile entry which should prove valuable in work other than germ-free life production where absolute protection of the individual is necessary. We wish to increase work on this particular project.

The work in biochemistry and nutrition has continued to produce information of fundamental importance. Emphasis should be placed on the basic LOBUND program in this field which involves the study of germ-free animal metabolism. This work has been held to a minimum because of the great demand for the animals by other agencies leaving us with very few for our own studies.

The work as reported on total body radiation indicates a pattern of considerable importance to the radiation sickness studies. We have also initiated in this period the studies on the physiology of Cesarian-born laboratory animals. There is a lacunal in the literature both with respect to the nutritional requirements of the suckling young and the physiology of the hand-fed germ-free mammals. This program is in its initial stages and will continue to be developed.

It has become increasingly necessary to establish routine histological facilities. We have studied this problem and are initiating a program to this end.

One of the interesting developments, with respect to the growth effect of antibiotic-fed animals, was the morphological changes with respect to organs and systems in the animal. This work opens up the possibilities of studying the direct effect of antibiotics on the animal system rather than on the microbial systems which ordinarily are found in conventional animals.

The work in virology is beginning to crystallize. Here we are working in a totally new area inasmuch as nothing is known with respect to the effects of viruses in germ-free animals. It should be noted with considerable interest that we have been successful in isolating a virus from infectious hepatitis sera. Should this eventually prove to be the virus of infectious hepatitis, the use of germ-free animals would be more than justified because up to the present time no experimental animal existed for this purpose. We would like to continue to press the basic aspects of the virology program but it is unlikely we will be able to do so with the demands to turn what few animals we are allotted into more applied virus problems.

The liver necrosis problem with the Army through ONR continues with the finding to date that germ-free rats do not show liver necrosis as do control rats fed on the Gyorgy diet.

The collaborative project on the etiology of dental caries with the University of Chicago (Zoller Memorial Clinic) is ready for publication. We are at the moment gathering up a few loose ends before submitting this work to the scientific press.

The immediate over-all problem before us is to continue to plan toward expansion of LOBUND facilities and to strengthen the basic research program. The latter problem will mean that we must withdraw from some of the applied work pressed upon us. This, however, is more than justified with respect to the future. Unless there is a well of basic information from which to draw, little is gained.

Respectfully submitted:

James A. Reyniers
Principal Investigator
Director, LOBUND Institute

University of Notre Dame

LOBUND Institute-ONR Semi-Annual Report

31 July 1952

For Period 1 January 1952 to 31 July 1952

CONTRACT: N6-ori-83, T. O. III NR:131-067

Prof. James A. Reyniers, Principal Investigator
Director, LOBUND Institute

Sections Reported

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I. ADMINISTRATIVE

(Compiled by Robert F. Ervin, Task Administrator
and Assistant Director, LOBUND Institute)

A. Personnel:

As of 30 June 1952 48 persons were employed on this Task Order. The salaries of 39 were paid out of the contract and 9 were paid by the University of Notre Dame. The total number employed 31 December 1951 was 44.

Turnover continued high in about 15% of the total jobs. Personnel in the other 85% have been stabilized. Typing, animal feeding and the diving job have suffered the greatest turnover. Steps are being taken to overcome this problem. As the average age and experience of this staff has increased, job turnover in the 15% has caused less disruption to the overall research effort.

B. Physical Plant:

The air conditioning system in the Germ-Free Life Building has been partially rebuilt so that its efficiency is much greater. Safety controls have been and are being installed to prevent accidents such as happened in November, 1951 with its disastrous effect on the germ-free animal colony.

Steps have been initiated to install an enlarged histological technique section in the radiation wing of the Germ-Free Life Building Technicons supplied through ONR by NMRI along with new laboratory furniture supplied by the University, will soon be in operation.

C. Publications:

Science and Society, a symposium edited by R. F. Ervin, was published in March, 1952. It includes a paper by Admiral Thorvald A. Solberg, USN (ret.) entitled "Basic Research and the Navy". Another paper "Germ-Free Life and Dental Research" by Dr. J. Roy Blayney presented some of the collaborative findings under this Task Order.

A new textbook Bacteria by Thomas and Grainger, published in 1952 by Blakiston carries a large illustrated section on the germ-free life research program at LOBUND Institute.

D. Colloquium:

As stated elsewhere in this report, a colloquium was held at LOBUND Institute 4 June 1952 on the subject "Studies on the Growth Effect of Antibiotics in Germ-Free Animals". Some 85 registered and attended the two sessions. The proceedings, papers and data presented have been forwarded in a special report to ONR Washington.

E. Collaborative Programs:

No new collaborative programs have been initiated and the technical progress of the programs are reported in the individual research sections of

this report. However, certain other aspects should be mentioned.

Dental caries (Zoller) - Captain James A. English of ONR Washington attended a meeting here 8 July 1952 with Dr. Frank Orland of Zoller and our research staff. The present status of this project and of the forthcoming publication were gone over in detail.

Liver necrosis (Army Medical & Univ. of Pennsylvania) - Dr. Martin Forbes of Dr. Paul Gyorgy's group is now stationed at LOBUND Institute and working on this problem.

Amebiasis (NIH) - Mr. Bruce Phillips of NIH will come to the Institute in July to carry out additional experiments on this project.

II. APPARATUS AND TECHNIQUES

A. Germ-Free Production
(Compiled by B. A. Teah, Chief of Germ-Free Life Production)

1. Apparatus and Techniques

Improvements in apparatus design and technique to better produce and rear animals germ-free, are listed as follows:

a. A Minneapolis-Honeywell alarm system, governing a high and low stage for both temperature and germ-free air lines, has been installed and integrated to give a warning signal for any approaching danger level in either building being operated.

b. A complete segregation of mammalian and chicken work was effected in this period, i.e., all chicken work is carried out in the Biology Building and all mammalian work in the Germ-Free Life Building.

c. Three new panels for sterilization of cages and claves were installed.

d. The majority of valves in use are now Hills-McCanna type diaphragm valves, replacing packing stem style. This change affords a tighter valve and a safer operation.

e. All claves before loading for sterilization are now being sprayed with a disinfectant as a precautionary measure in routine operation.

f. An inter-communication system has been installed between the Biology and Germ-Free Life Buildings thus giving a speedier and more controlled operation.

g. At present a visible ball float regulator is being tried for eventual use on all germ-free cages. Its purpose would be to continually show and govern the optimal air flow into the germ-free units.

h. An all neoprene glove for use on the germ-free cages designed

and formulated by Dewey and Almy Co. is now being used on some germ-free cages. These gloves at this time have given very satisfactory results. They withstand sterilization very well, age very slowly and possess very excellent resiliency. It is believed that with this glove, replacing the American Anode type, the number of glove mishaps and contaminations will be considerably reduced.

i. A shower stall for quick washing of interior and exterior of all cages was installed.

j. The majority of cages in the Germ-Free Life Building are equipped with new oil traps as previously reported.

k. A dual lighting system has been installed in all chicken cages, so that each group will receive equal lighting.

2. Animal Production

In this six month period, the work was handled in the following equipment: 6 series 50 cages, 4 series 20 cages, 10 series 100 cages, 9 series 200 cages, 2 operating cages, 2 examining units and 1 X-ray cage, - a total of 34 cages; an increase of 6 cages in the past six months.

In the July 1951 to December 1951 report, it was stated that the number of hand-fed rats produced was very low. In this period the number of experiments to rear hand-fed rats has decreased over that reported time - this being mainly due to lack of equipment and personnel and also less demand on this source because of the germ-free colony. It must be stated, however, that with the experiments attempted, we would have reared at least 20% and added them to our stock colony but glove breaks caused contamination of all animals we had successfully brought up to 20 days of life.

Our stock colony of germ-free animals has increased markedly from the previous report. At present we have a colony of 120 animals - this in spite of the serious loss of most animals in November, 1951.

The chicken work previously reported as routine has successfully continued and increased due to increase in number of cages allocated to this work.

In the past six months, turkey work in Germ-Free Life Production has progressed so well that it is now considered as routine as the chicken work.

B. Germ-Free Colony Apparatus
(Compiled by P. C. Trexler, Assistant Director)

The operation of the germ-free colony tank during the past year has indicated that the formaldehyde entry bath is the least desirable feature. This bath complicates the entry procedure and garment design. In addition, the diver introduces a considerable amount of formaldehyde gas with each entry. Preliminary experiments with flexible vinyl structures indicate that a 25% formalin solution sprayed on sterilizes rapidly and can be readily neutralized and washed off. Thus it should be possible to substitute a formalin spray for the bath as far as sterility is concerned. The bath also serves as a tight entry port. A simple liquid seal door has been designed which serves as an entry port.

At the present the full size model of the germ-free colony tank in the Biology Building is being refitted to test the above design. It is felt that this tank can be put into germ-free production.

III. BIOCHEMISTRY AND NUTRITION
(Compiled by T. D. Luckey, Chief Biochemist)

A. Introduction

During the past six months the Division of Biochemistry and Nutrition have spent the most energy on the problems of the rat biochemical survey, the chick vitamin survey, the effect of antibiotics on the growth of germ-free chickens and the preparation of LOBUND Reports No. 3. The work presented was prepared by: J. Pleasants, Mrs. M. Beaver, Miss A. Pappas, Mrs. L. MacAllister and Miss L. Takacs.

B. Biochemistry

1. Germ-Free Rat Survey:

The material for the germ-free rat survey was taken last fall. This material has been processed in the laboratory and conventional rats are being maintained to the proper age for a control group. Some of this latter group have already been sacrificed but the data have not been processed. Details of this project will be reported later.

2. Germ-Free Chick Survey:

Part of the detail of results in this project was reported previously (Semi-Annual Progress Report, July - December 1951). Further processing of the data from bantam chicks reveals some very interesting phenomena with respect to the growth of the animal. Analysis of the cecal contents (wet weight basis) of White Wyandotte Bantam chicks (Fig. I) indicated the young germ-free birds had a much higher riboflavin content than that found in conventional birds. This difference disappeared when the chicks became 4-6 months old.

The amount of liver tissue functioning for the birds was compared as mg dry liver per 100 gm body weight. This comparison (Fig. II) indicates the germ-free birds have less functioning liver per unit of body weight than do the conventional chicks. This effect is marked in the 30 day old group and appeared to become more noticeable after the age of about 200 days.

Examination of the liver ash on the same basis (Fig. III) indicates the germ-free chicks have lower quantity of ash in their liver per unit of body weight than do the conventional chicks. This difference is increased with age since the germ-free chicks have a continual decrease up to 5 months and the conventional birds appear to increase the amount of liver ash per unit body weight after 5 months.

The other factor investigated showed that an interesting pattern was liver pantothenate. As seen in Fig. IV, the store of liver pantothenate per 100 gm body weight continually decreases as the germ-free birds grow older while the conventional bantam chicks show no consistent pattern.

Fig. 1

Riboflavin in Cecal Contents (Mcg gm Wet Basis) of Bantam Chicks

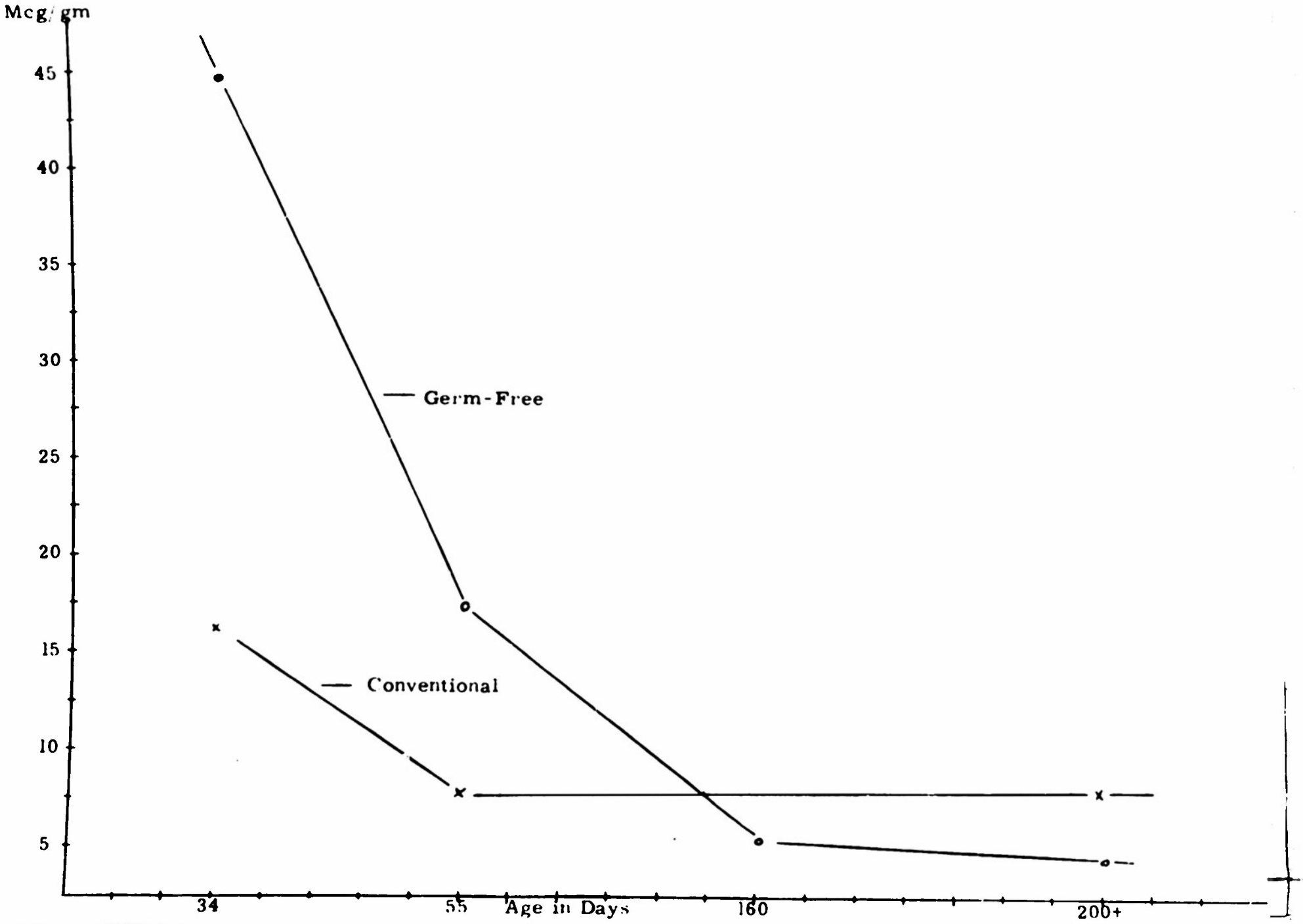


Fig. II

Mg Liver Dry Weight per 100 gm Bantam Cockeral

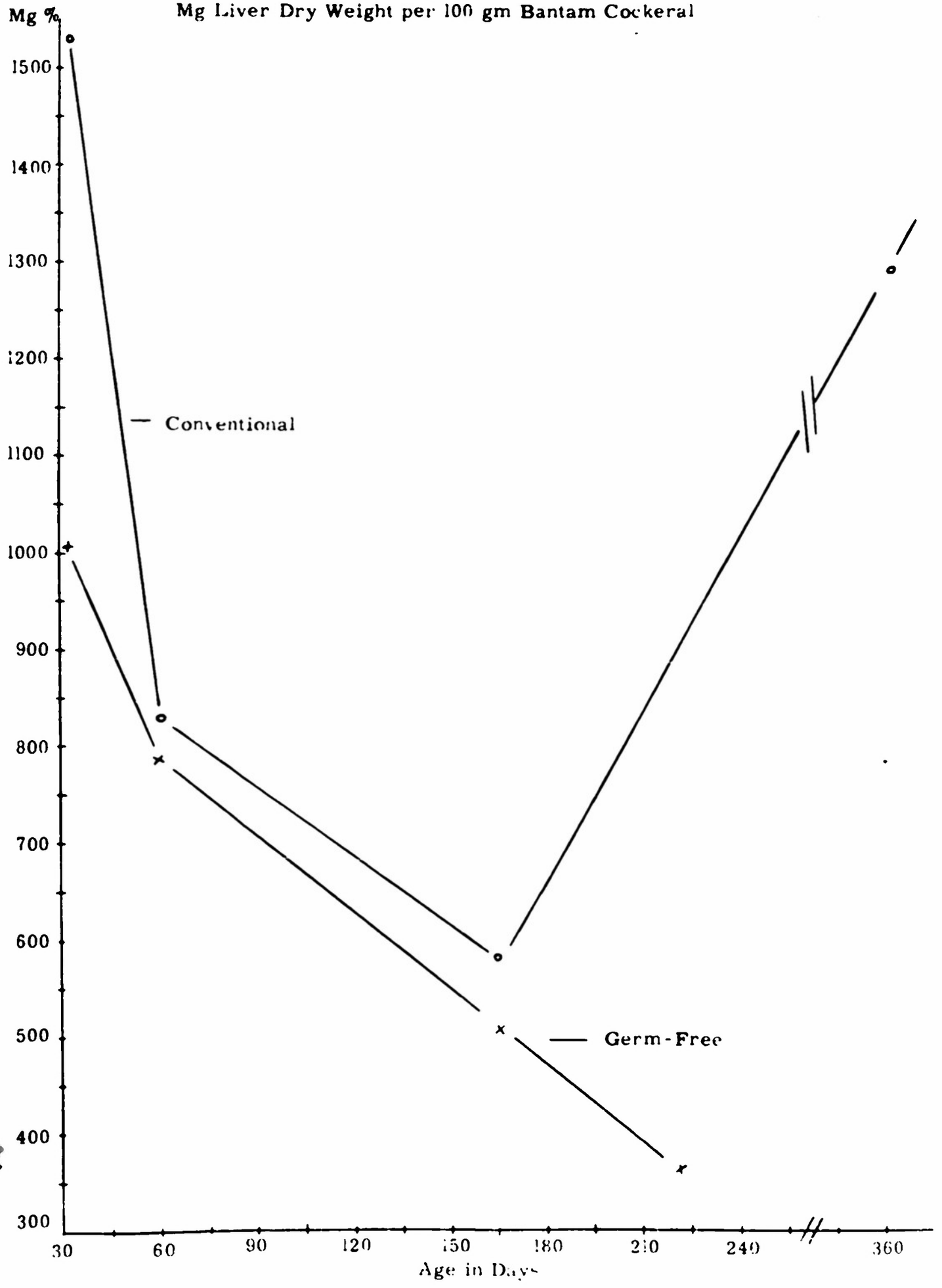


Fig. III
Mg Liver Ash per 100 gm Bantam Chicks

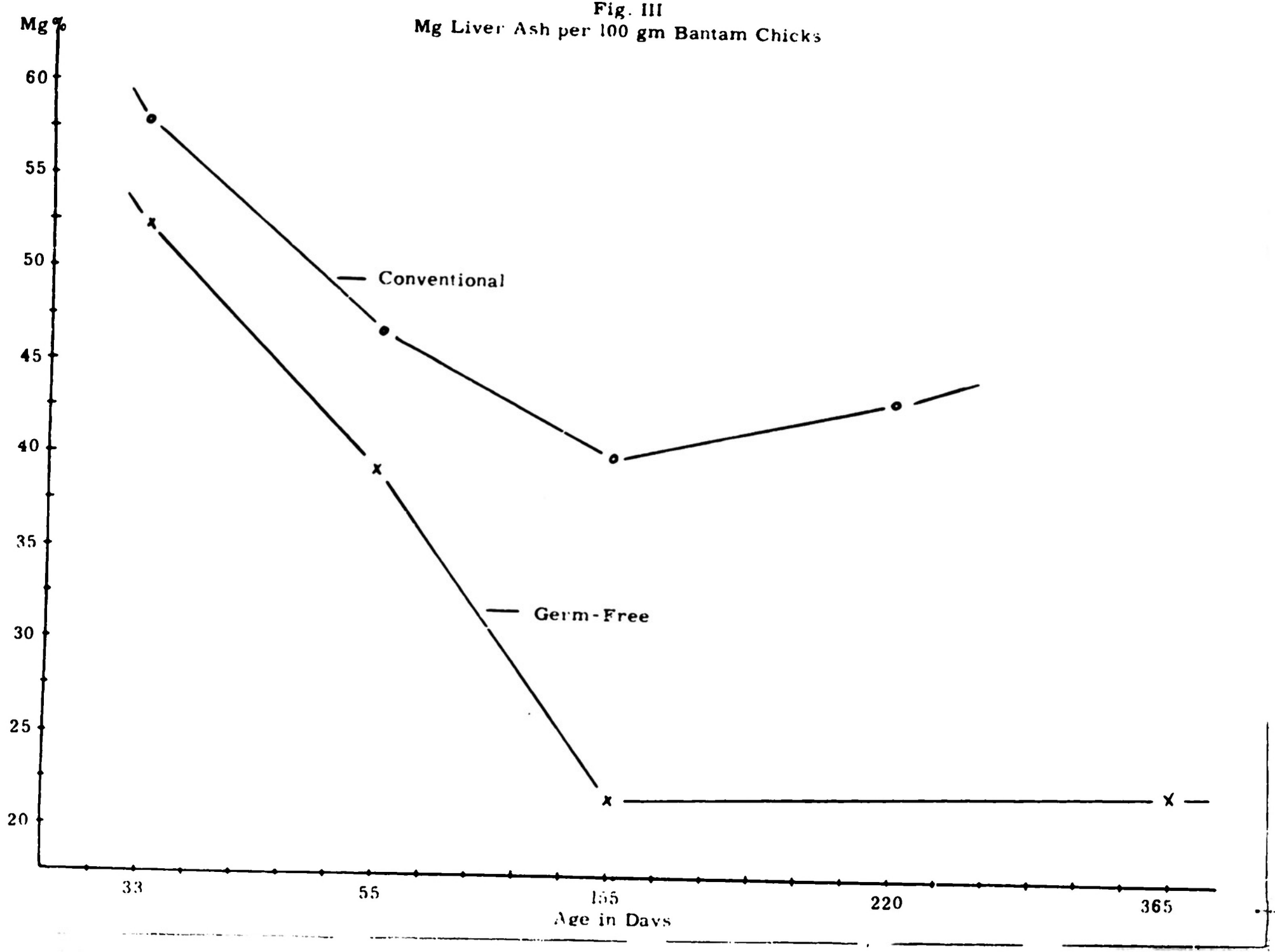
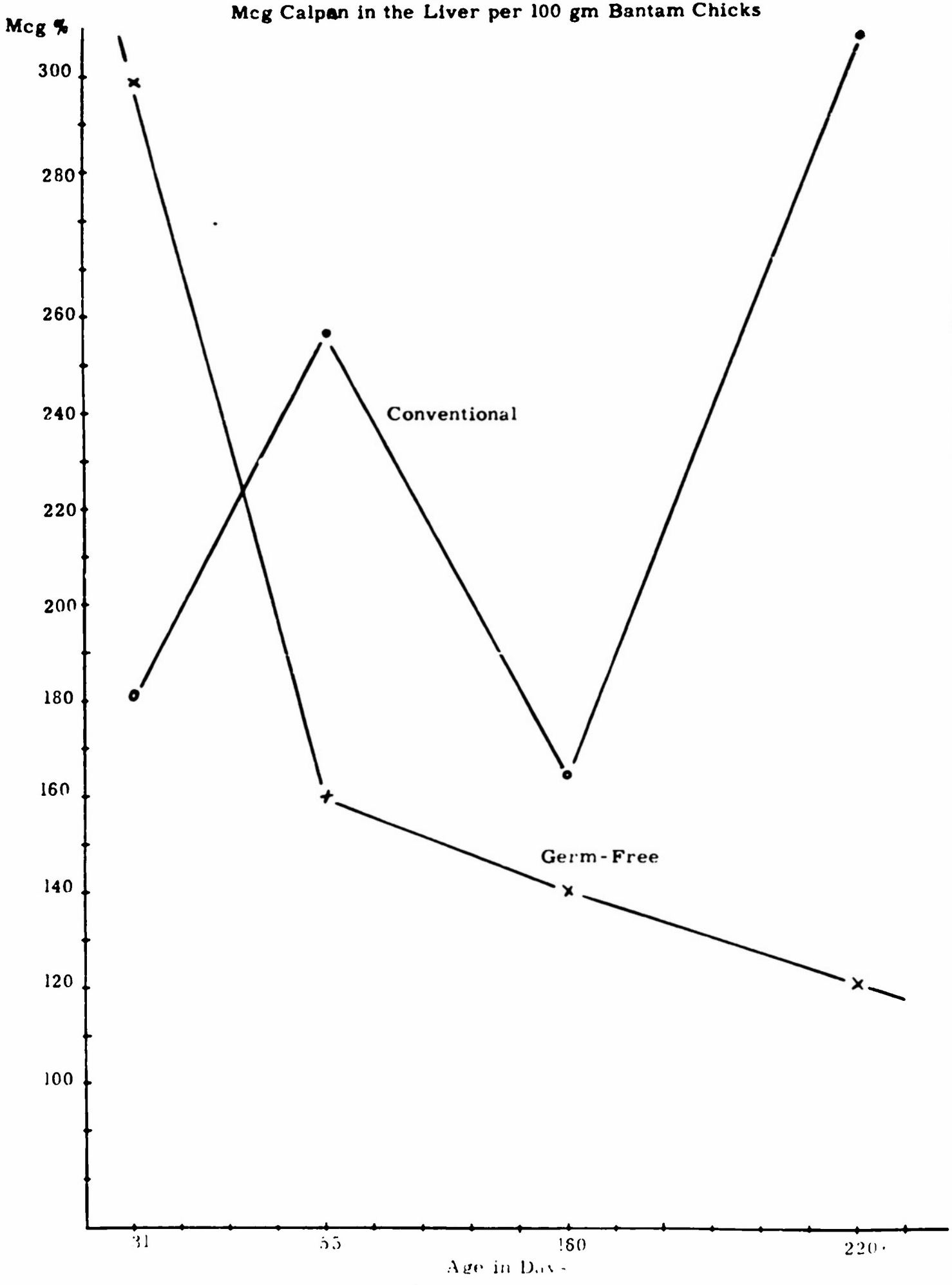


Fig. IV

Mcg Calpan in the Liver per 100 gm Bantam Chicks



These data are given to exemplify the differences between germ-free and conventional bantam chicks. The similarities between the two groups are more numerous if less dramatic. Speculation as to the function of these differences in metabolic pathways is purposely withheld.

C. Nutrition

1. Chick Survey

The following experiments have been run in a survey of the nutritive requirements of germ-free chickens.

Diet	No. Expts.	Conclusions
Complete-practical	Ca 10	Need high protein
Complete-synthetic type	Ca 20	No liver, yeast, etc., needed
Vitamin-A deficient	2	Germ-Free chicks need Vit. A
Vitamin-E deficient	1	Current experiment
Vitamin-K deficient	10	Germ-free chicks need Vit. K
Thiamin deficient	4	Germ-free chicks need thiamin
Riboflavin deficient	2	Germ-free chicks probably need this
Niacin deficient	5	Germ-free chicks probably need this
Folacin deficient	4	Germ-free chicks need folacin
Biotin deficient	3	Germ-free chicks need biotin

It is of interest that a previous report stated that no requirement for Vitamin K could be demonstrated in germ-free chicks. It has recently been shown to be required several times and the early failures have not been satisfactorily explained.

The general picture of these qualitative experiments is that there are no big nutritional differences between germ-free and conventional White Wyandotte Bantam chicks.

2. Rat Survey:

One preliminary experiment should be summarized because it has rather far-reaching implications. Two germ-free rats, available for the nutritional survey, were fed diet L-283 (see Table I for composition). One of these died soon after biotin therapy was inaugurated. Two conventional rats were used as controls. All rats were maintained in metabolism cages. The germ-free rats exhibited symptoms which were interpreted as a severe biotin deficiency. They were given injections of biotin and biotin was added to the diet. The biotin excretion increased about 40 times in the feces and about 1000 times in the urine. This was to be expected since 50% is a high dietary level for biotin.

TABLE I

L-283 RAT

Composition
November 21, 1950

CONSTITUENT	AMT./100 g diet
Casein, Labco	25 gm
Starch, corn	59 gm
Cellophane spangles	2 gm
Salts L-II	6 gm

TABLE I (Cont'd)

CONSTITUENT	AMT./100 g diet
Corn oil	5 gm
Vitamin A	400 I.U.
Vitamin C	200 mg
Vitamin D	50 I.U.
Vitamin E (alpha)	25 mg
Thiamin	5 mg
Riboflavin	3 mg
Pyridoxine	2 mg
Pyridoxamine	0.4 mg
ca pantothenate	30 mg
Nicotinamide	5 mg
Niacin	5 mg
p-aminobenzoic acid	5 mg
Choline chloride	200 mg

Most unexpectedly in the germ-free rat, the folic acid concentration rose about 5 fold (fifty times based on one previous determination) in the urine and with a 10 fold increase in fecal concentration in a period of 4 months. This phenomenon was much less apparent in the conventional rats. Water intake and urine output tripled in the germ-free rats and doubled in the conventional animals. The effect of these factors upon daily excretion of folic acid is seen in Figure V. It should be emphasized that there was no change in folic acid content of the diet. The figure indicates an increase in folic acid excretion by the germ-free rats upon the addition of dietary biotin. This excretion rose to 20 times its original value in a period of four months.

The possibility of biosynthesis of folic acid in the rat is obviously implicated. This limited experiment suggests that biotin is required by the germ-free rat; that folic acid is not required - but is actually synthesized by the tissues in the presence of biotin (and not in the absence of biotin). Confirmation of these results would certainly change our attitude concerning the indispensability of certain vitamins and may alter the basic concept of vitamins generally.

3. Rearing Rats

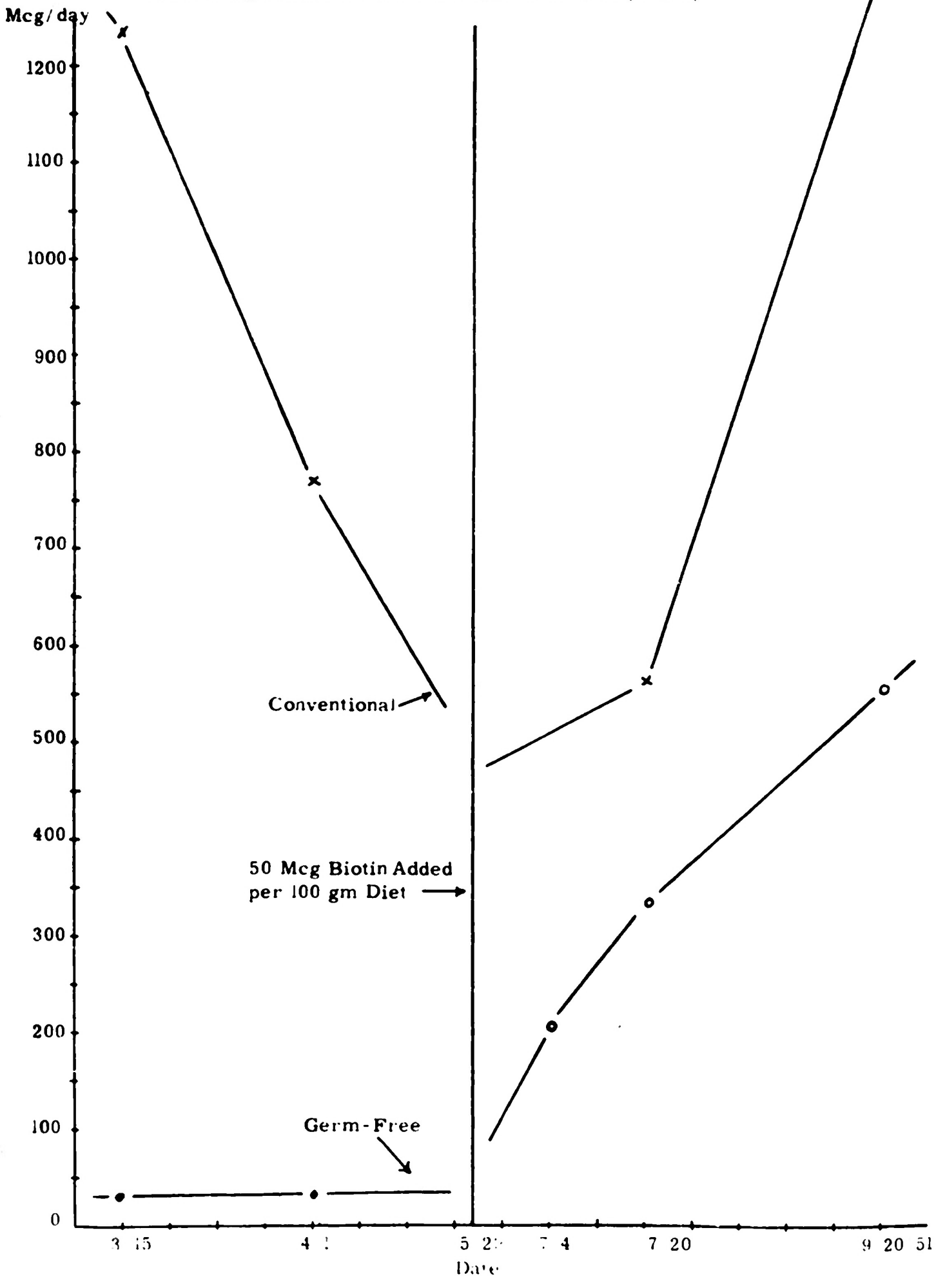
We have previously presented our observations on a "seasonal factor" in the successful rearing of handfed newborn rats. It now appears that this factor may be better considered as a cyclic factor since it cannot be correlated with seasons, temperature, light, etc. We seem to be emerging from a particularly long "bad" season.

4. Antibiotics in Germ-Free Chicks

We have worked for almost 2 years on this problem in an attempt to answer the simple question, "Will dietary antibiotics produce a growth response in germ-free birds?". Our results were presented to interested people (invited by personal invitations and through announcements in accepted scientific journals) in a colloquium held at Notre Dame on June 4th. Since the

Fig. V

Folacin Excretion in Rats Fed Biotin Low Diet (L-283)



presentations have been submitted to ONR as a special report, it is proposed to summarize the results very briefly at this time. As seen in Figure VI, the usual growth response was obtained at LOBUND using conventional birds fed autoclaved diets with and without sterile antibiotics from the same batch as that fed the germ-free birds. The results with the conventional chicks gave an average of 4% better growth with 50 mg of terramycin per Kg of feed and 7% better growth with 50 mg procaine penicillin per Kg of feed (practical mash with a high protein and vitamin content). The conventional turkeys grew 7% faster when 50 mg procaine penicillin per Kg feed was added. In contrast it is noted that no growth response was seen in germ-free chicks or poults when different antibiotics or sulfasuxidine were added to the diet. This result implies that the mechanism of action of the antibiotics in promoting the growth of conventional animals is either through the vehicle of the microflora of the host or through a system of the host conditioned in some way by the microflora.

Biochemical analyses of germ-free and conventional chicks fed these drugs are presented (Figs. VII, VIII, IX, X, and XI) in a manner that permits comparisons. These data indicate an interesting change in the metabolism of folic acid, biotin and vitamin B_{12}. The change in liver ash may have been anticipated from a report in the literature on the effect of feeding sulfa drugs.

D. Collaborative Problems

1. Zoller Dental Clinic Collaboration in the Use of the Germ-Free Technique in the Study of Dental Caries.

The first phase of this work is completed and is being prepared for publication. The results are briefly: 13 handfed germ-free rats showed no lesions when fed Diet L-128 (composition given previously); all of 16 conventional rats fed the same autoclaved diet for the same period of time developed lesions; when the handfed germ-free rats were taken to the conventional stockroom at weaning(20-30 days) and then placed on the cariogenic diet for 5 months, 6 of ten had carious lesions, the other four had none. When normally-born germ-free rats (2nd and 3rd generation) were fed a somewhat different condition, these rats again showed no lesions while 17 of 20 conventional rats developed lesions.

Preliminary experiments on the second phase have been reported previously. Briefly stated, the results were variable with about 1/3 of the rats showing some lesions. The current experiment in this phase involving 12 germ-free and 8-12 conventional rats all fed diet L-128. Four of the 12 germ-free rats remain germ-free, eight of them were inoculated with L. acidophilus L-#456. Four of these were subsequently taken to conventional animal quarters exposed to the usual polyflora. It is hoped that data from this experiment will be available for the next report.

2. University of Pennsylvania Medical School:

Collaboration with Dr. Gyorgy: The susceptibility of germ-free animals to diet induced hemorrhagic liver necrosis.

Fig. VI

Averages of Antibiotic Experiments (With Sterilized Diets and Antibiotics)

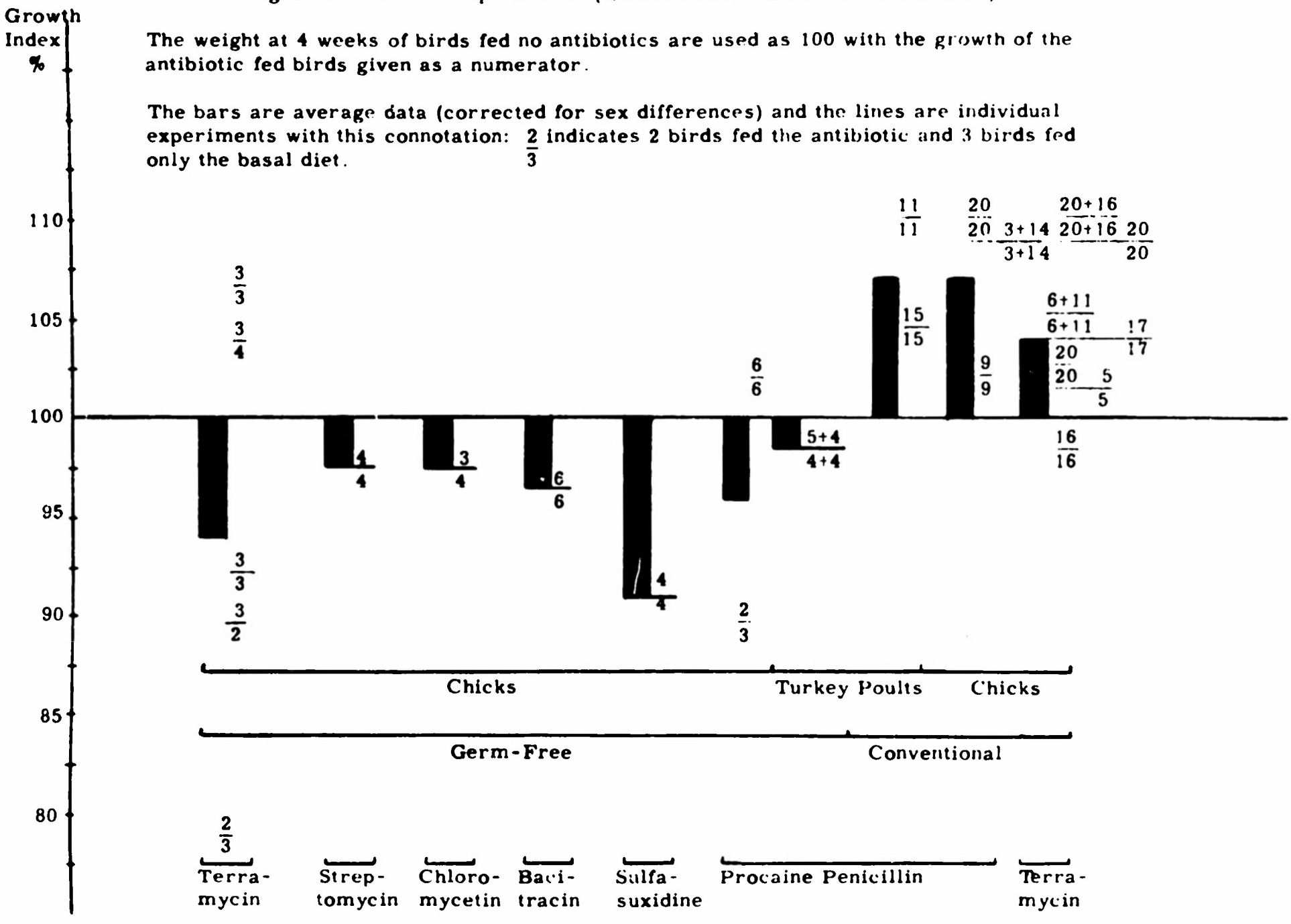


Figure VII

Biochemical Comparison of Germ-Free to Conventional Chicks Fed Diet L-289

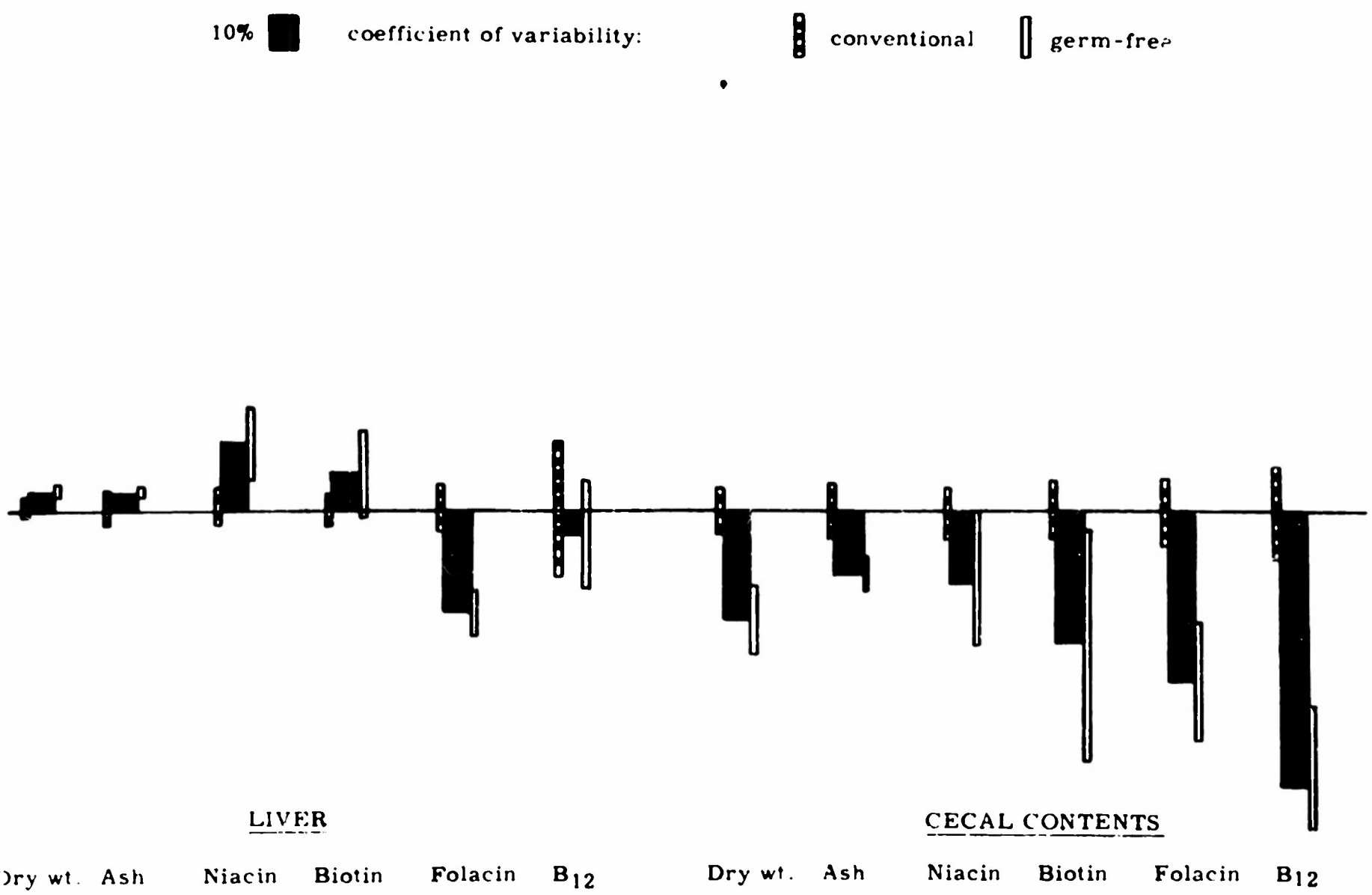


Figure VIII

Terramycin - Germ-Free

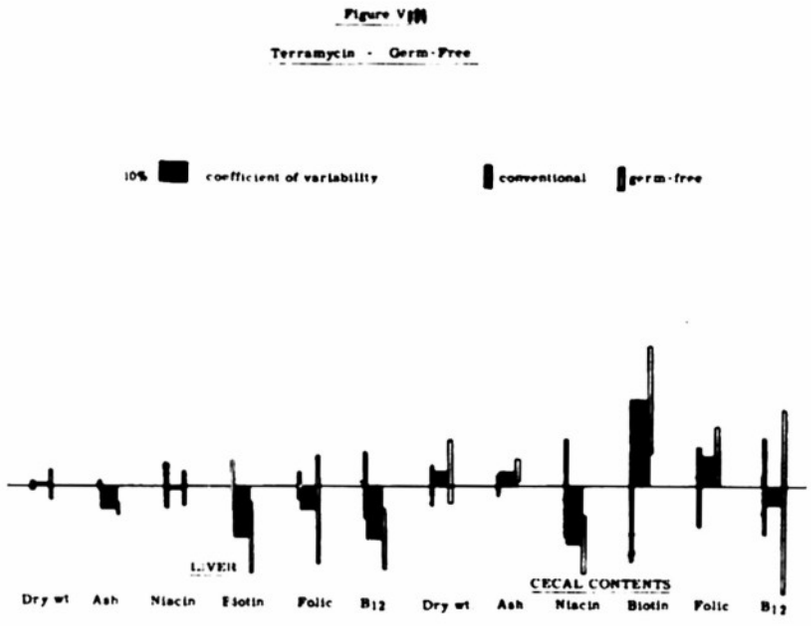


Figure IX

Terramycin - Control

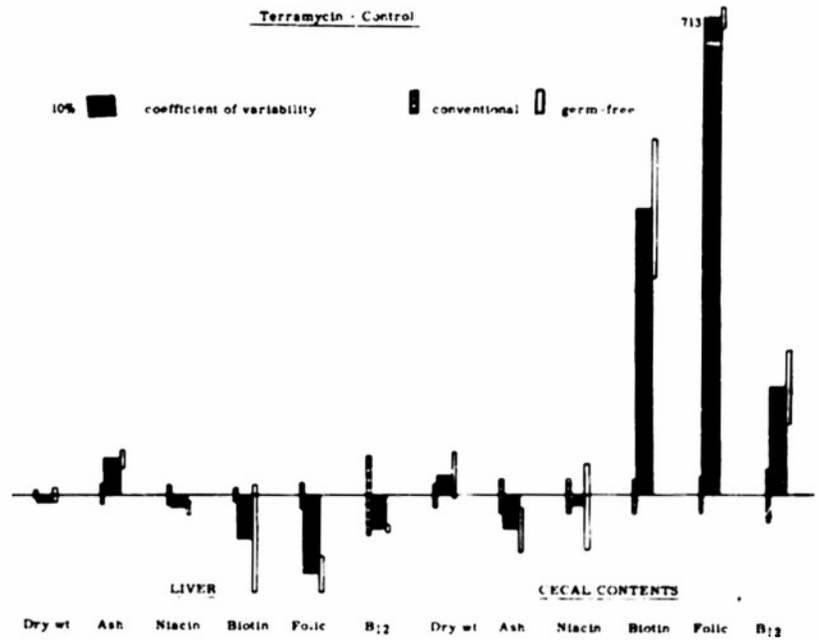


Figure X

Streptomycin - Germ-Free

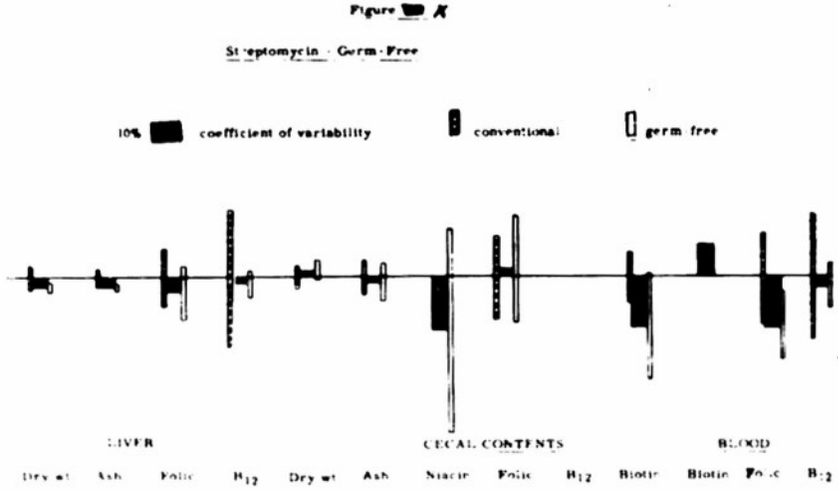
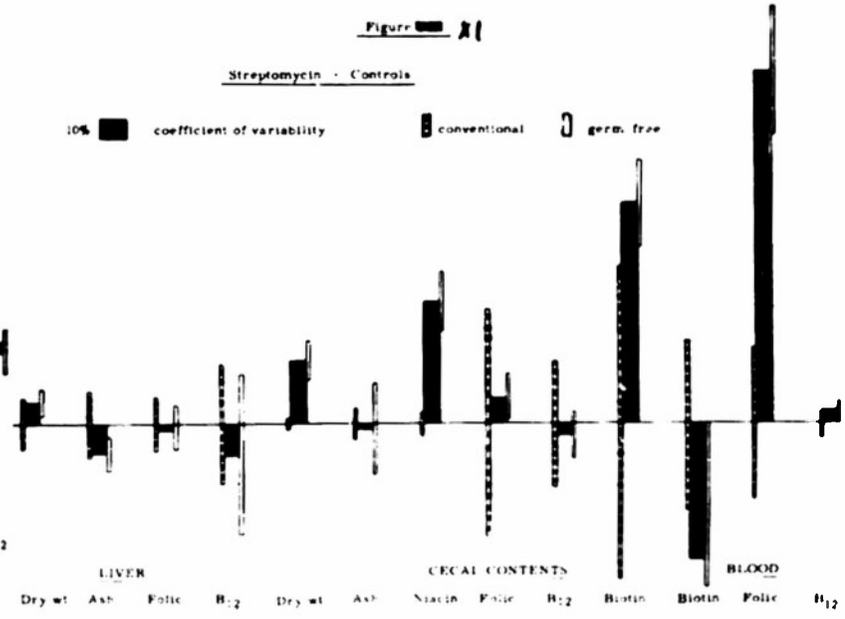


Figure XI

Streptomycin - Controls



Three experiments have been run which indicate the germ-free rat is more resistant to this syndrome than the conventional rat fed the same autoclaved diet. A fourth experiment is underway which may prove the point indicated.

E. Apparatus and Methods

1. Merry-Go-Round Cage

A cage has been designed and built for rearing experimental groups of chicks in such a manner as to minimize differences in environment. The cage is arranged as three pies stacked on a stand which revolves 1/10 r.p.m. Each pie has six segments, which are equal in size, shape, light and space to each other by virtue of the revolution. Chicks in different tiers are of course, in different environments, so only six groups on the same level are strictly comparable. The unit is now being tested to determine whether this standardization of environment will produce a practical reduction in variation between experimental groups of chicks.

2. Germ-Free Turkeys

A noteworthy accomplishment during the past six months has been the adaptation of the apparatus and methods for rearing chicks to rearing turkeys. Suffice it to say no particular problems are involved and we have successfully used turkeys in critical experiments on the effect of antibiotics on growth.

F. Future

The next six months may well be entitled "Germ-Free Rat Survey Period". Other areas to be rounded out should be the rat milk analysis program and the antibiotic problem. Problems in progress indicate not only those mentioned herein but the decontamination of adult animals, the use of carboxide to sterilize liquid diets, the protein components of germ-free blood serum and development of the small germ-free jar.

In the more distant future should be the installation of a micro-chemistry laboratory and more active use of radioisotopic techniques.

IV. BACTERIOLOGY AND SEROLOGY
(Compiled by Morris Wagner; Chief Bacteriologist)

A. Bacteriological Testing of Germ-Free Cages

This function of the bacteriology laboratory continues as a service to various LOBUND projects being run in germ-free equipment. The work includes:

1. The systematic check on bacteriologic sterility (germ-freeness) of experimental animals being run under germ-free conditions.

2. The systematic check on animals being run in germ-free type equipment to which known strains of bacteria have been added (e.g., rats monocontaminated with Lactobacillus #455 for the dental caries project). This includes checks on the purity or stability of the purposely inoculated strain as well as the continual search for accidental contaminants which might possibly be introduced via some accidental failure in the operation of the equipment.

3. The determination of the type of contamination which might accidentally occur in experiments originally scheduled to be run germ-free. This phase is important for two main reasons:

a. The type of contamination sometimes furnishes a clew to the portal of entry of the accidental contaminant.

b. Accidentally contaminated animals can be used in certain projects (e.g., accidentally contaminated animals have been used in the X-ray experiments). In such cases, we attempt to identify the organism in order to define our experimental conditions more fully.

With respect to the above functions of the laboratory, the amount of work involved has necessarily increased with the increase in the number of germ-free cages being run (including the transfer of the germ-free colony apparatus, "Big Tank", from research to production). It was therefore necessary to divert considerable energy to fulfill these functions during the expansion of facilities and apparatus.

As of July 2, 1952, we have added Mrs. Marjorie R. Osterhout to the staff to assist in the germ-free testing service.

B. Bacteria in Feces of Germ-Free Animals

That bacteria or bacteria-like forms can be seen by the microscopic examination of stained fecal preparations made from germ-free animals has been discussed in previous progress reports to ONR and in LOBUND Reports No. 2 (1949) p. 99 - 103. These forms can not be cultured by various culture procedures. They are believed to be dead bacterial cells which are in the diet and tend to accumulate in the feces of germ-free animals as dead undigested transient cells.

Our attention has been refocused on this phenomenon because in the past six months to one year, the bacteria-like forms seen in the feces of adult germ-free rats have changed quantitatively as well as qualitatively.

Quantitatively, more bacteria-like forms are encountered when Gram-stained smears from feces are examined. These forms represent an increase over what we previously encountered but generally, the numbers are still less than that seen in the feces of animals from which viable organisms can be cultured.

Qualitatively, the majority of organisms appear much like spore-formers and indeed free bacterial endospores can be seen in the feces; but, cultures are negative.

Generally, the mixed diet (L-128) fed to these animals does not show these spore formers upon microscopic observation but this may be due to the difficulty in concentrating microbial forms from the diet. Similarly, examination of the individual dietary ingredients has not revealed any particular ingredient as a contributing source of the spores. In this respect, our methods of observation may be inadequate to detect the organisms, particularly if they were to come from the oily or large particle constituents of the diet.

With respect to culture procedures on the feces from the germ-free rats, we have tried the following, all with negative results:

1. 5% blood agar plates at room temperature 37°C and 55°C.
2. Same as (1) but under anaerobic conditions.
3. Same as (1) and (2) but using Brain Heart Infusion Agar without added blood.
4. Brain Heart Infusion Broth and Fluid Thioglycollate Medium at the three aforementioned temperatures.
5. Incubation of the fecal material itself to note any increase in numbers where the feces themselves serve as the sole source of nutrients.
6. Inoculation of feces onto the chorio-allantoic membrane or into the yolk sac of embryonated chicken eggs.

On the basis of the presence of spores in the feces, we have investigated these forms from the standpoint of spore "dormancy" described by Foster and Wynne and others. Heat shock of the spores as recommended by Mefford and Cambell to overcome dormancy has not been successful.

On the basis that inhibitory substances may be present in our media which interfere with growth or spore germination, 0.1% soluble starch (Olsen and Scott) or 0.1% activated carbon (Pollack) have been added to the media with no success.

Our present belief, backed by much evidence, is that these forms are dead transient cells coming in with the food. However, if they are viable, but need environmental and nutritive conditions as found in the

Foster and Wynne, J. Bact. 55:623 (1948)
Mefford and Cambell, J. Bact. 62:130 (1951)
Olsen and Scott, Nature 157:337 (1946)
Pollack, Brit. J. Exp. Path. 28:295 (1947)

gastro-intestinal tract in order to grow, then a critical experiment can be set up to show transmission and establishment of these forms in germ-free animals showing no previous history of these microscopic forms.

C. Bacteriological Aspects of LOBUND X-Ray Radiation Studies

As in the previous progress report, there has been no systematic bacteriological study of the X-rayed rats since, for the most part, we have been studying dose-mortality rates and have not wanted to influence results by performing cardiac punctures on the animals. A few tests were run on the heart blood of animals maintained in germ-free type equipment but having a limited flora. Results are recorded in the main body of the X-ray reports in Section V of this report.

D. Isolated Control Animal Colony:

The isolated Control Animal Colony ran from November 6, 1950 to February 6, 1952. The following list of organisms and the date of their detection is shown in the following table.

	Organism	Date of Detection
1.	Micrococcus pyogenes var. albus	Nov. 6, 1950
2.	Micrococcus conglomeratus	"
3.	Bacillus coagulans	"
4.	Bacillus pumilis	"
5.	Penicillium species	"
6.	Micrococcus epidermidis	"
7.	Bacillus species (terminal spores)	"
8.	Yeast	"
9.	Fusiforms*	"
10.	Escherichia coli	Nov. 10, 1950
11.	Lactobacillus species	"
12.	Aerobacter aerogenes	Nov. 29, 1950
13.	Pseudomonas species	"
14.	Proteus species	Dec. 13, 1950
15.	Streptococcus fecalis	Jan. 31, 1951
16.	Streptococcus liquefaciens	Feb. 21, 1951
17.	Streptococcus salivarius	March 7, 1951
18.	Spirilla*	July 10, 1951
19.	Escherichia intermedium	Sept. 26, 1951
20.	Paracolobactrum aerogenoides	Nov. 28, 1951
21.	Alcaligenes species	Feb. 6, 1952
22.	Chilomastix*	Feb. 6, 1952

* These organisms seen in wet mounts prepared from cecal contents but not cultured.

The Animal Colony was discontined on February 6, 1952 when the protozoan flagellate Chilomastix was detected in the colony. Our original objectives had been to maintain animals free from protozoans, salmonellosis, worm and insect infestations.

The source of Chilomastix was undoubtedly from a wild mouse which gnawed its way through a wall into the isolated room. The rats in the isolated colony were still free from salmonellosis and worm and insect infestation according to our tests.

E. Sterility Testing of Antibiotics

We have previously reported that activated carbon will effectively inactivate antibiotics so that sensitive organisms can grow out in carbon-containing media containing ordinarily inhibitory antibiotic levels.

Our next step was to determine whether various organisms are capable of developing in media containing 1% carbon. We also wished to check whether growth in the carbon medium could initiate from small inocula. These two steps were necessary since we can generally suppose that a contaminated sample of antibiotic might contain a variety of contaminants and secondly that the contaminants may only be present in small numbers.

Twenty-three stock strains of bacteria representing 14 genera were checked for their ability to grow in the basal medium ± 1% activated carbon.

In all cases, growth in the presence of 1% activated carbon was equivalent to growth in the basal medium without carbon except for the members of the genus Streptococcus and Lactobacillus. In these latter genera, good growth was obtained in the carbon-free medium but little or no growth occurred in the presence of 1% carbon. Of those that could grow in presence of 1% carbon, growth could be initiated from inocula diluted 10^{-6}.

With regard to the synthesizing power and nutritional requirements of various bacteria, the lactobacilli and streptococci are generally classified as "requirers" whereas the other genera used are generally regarded as "synthesizers". Since activated carbon is an absorbing agent, it is probably making essential nutralites in the medium unavailable to the "requirer" group, thus interfering with growth.

Attempts to replenish a probable vitamin deficiency in the carbon medium by addition of Difco Yeast Extract up to 2% and a peptone-amino acid deficiency by addition of Difco Casitone up to 2% has not resulted in any appreciable increase in growth for the streptococcus-lactobacillus group.

Using Lactobacillus casei as a test organism, we have checked the level at which carbon interferes with growth in 0.1% steps from 0 to 1%. Serious interference with growth occurs at the 0.3% level and higher, whereas 0.1% carbon allows growth equivalent to the basal medium.

Thus, while our original goal was to develop the technique on the more convenient basis of a high (1%) carbon content - small volume medium, we shall have to increase the volume of the test medium so that the final carbon concentration is lowered to about 0.1%. These latter tests are pending.

F. Dental Caries Project (Collaboration of LOBUND Institute and Zoller Clinic)

The present phase of the dental caries project is the study of the role of lactobacilli in the caries process. The experiment, indexed 39D1-9,

consists of 12 germ-free littermate rats divided into 3 groups of 4 rats each and handled in the following manner.

Group A (4 rats)

Maintain germ-free throughout experiment on a cariogenic diet (L-128 + 5% dextrose water).

Group AI (4 rats)

Spent first 48 days germ-free and later contaminated with Lactobacillus #465. Rats to be maintained on cariogenic diet in the germ-free-type apparatus harboring the single type organism throughout the experiment.

Group AIB (4 rats)

Same as Group AI above except that after contamination with the lactobacilli, the rats were brought out of the germ-free type apparatus and put in the animal colony in order to acquire the animal colony flora.

All groups are current and will be examined for dental caries incidence after 150 days on the cariogenic diet.

From the bacteriologic standpoint, we are following qualitatively and quantitatively the incidence of lactobacilli in the oral cavity of the rats involved in Groups AI and AIB. Group A (germ-free)is not involved.

In Group AIB, we are determining quantitatively and qualitatively, the oral lactobacillus count as measured by growth of lactobacilli on anaerobic Tomato Juice Yeast Extract Agar (TJY agar). The lactobacillus count on TJY agar will be compared to the "total count" of oral bacteria as measured on a non-selective medium; namely, aerobic and anaerobic 5% horse blood agar plates.

With regard to the Group AI, these rats only harbor a single type organism, Lactobacillus #465. Therefore in this group, no qualitative differentiations are necessary, the Lactobacillus #465 count representing the total count of the oral flora.

At the termination of this study, the bacteriological findings will be correlated to the caries incidence.

A second objective in this work is to follow the survival or establishment of Lactobacillus #465 in the rats in Group AIB after they have been brought into the animal room environment and thus into competition with other bacteria.

The quantitative data is too meager to be reported at this time. Qualitatively, we have 4 different lactobacilli involved in the AIB group, including the #465 strain which still persists in the oral cavity of the rats after 26 days in the animal room environment.

G. The Bacteriology of the Cecal Contents in Chickens Fed Growth Promoting Levels of Antibiotic.

This work was done on the conventional starter-battery-housed control chickens used in conjunction with the germ-free experiments devised to study "The Effect of Feeding Antibiotics upon the Growth Rate of Germ-Free Birds". The bacteriologic objective was to observe any marked changes in the bacterial flora which might be an indication of the mechanism of action of the antibiotic growth promotants in conventional animals.

The relative changes in intestinal flora produced by feeding procaine-penicillin G are shown in the graph. The bars represent the relative changes which occurred in treated chicks at four weeks of age, using the untreated chick data as a basis of 1.

The greatest relative change was seen in the streptococci with approximately a 42 X increase in the treated birds. However, in terms of actual count the streptococci made up less than 0.8% of the viable total flora detectable by our culture procedures.

Lactobacilli showed a relative decrease in the treated group, due mainly to inability to detect two of four lactobacillus strains recoverable from the untreated group, but not found in treated birds.

Yeasts, micrococci, aerobic and anaerobic spore formers and bacteroids were also included in the study but were too few in number to merit consideration.

Terramycin treated birds gave essentially the same results.

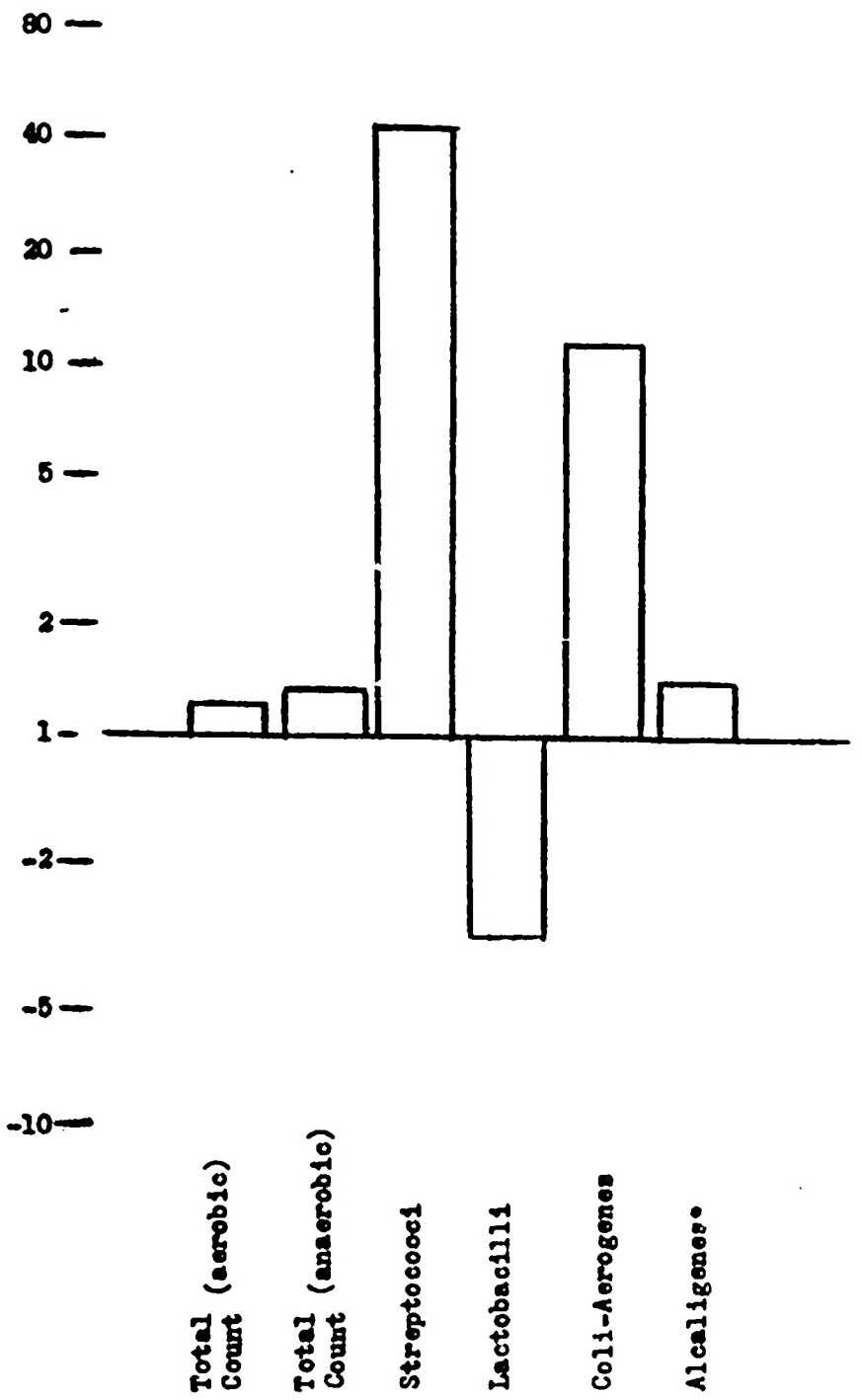
Observations were also made on wet mounts made from cecal contents of treated vs non-treated birds in order to observe what changes occur in those fecal forms which can be seen microscopically but which do not lend themselves to cultivation by our usual techniques. The following comments refer to these noncultivatable forms.

Fusiform organisms which make up a large portion of the visible but noncultivatable flora were not appreciably affected by penicillin (P) or terramycin (T) treatment. Large sarcinae seen in the non-treated and P treated group where completely absent in T treated birds. The reverse was true of protozoa in that Chilomastix was present only in the T group but not in the P or untreated groups. Lastly, long filamentous chains of organisms were much more prevalent in the T group although they also appeared to a lesser extent in the other groups.

While these visual differences did occur between P and T treated birds, the fact that they both are active growth promotants rules out these differences as being directly responsible for the growth response.

Our limited experience agrees with most publications in this field; namely, that bacteriological analysis of the feces has not revealed any drastic changes in the fecal flora to account for growth promotion.

The Effect of Feeding Procaine Penicillin G
on the Cecal Flora of Chickens



* Alcaligenes were the most predominant organisms cultured.

H. Serology Laboratory

Activities in the serology laboratory were greatly curtailed during the past six months due to loss in personnel and pressures due to increased activity in the bacteriology laboratory.

Miss Helen Budeit left us in March 1952 and we were unable to secure a replacement for her until July 1, 1952. During the first months of the year, Miss Budeit's services were also often required in the bacteriology laboratory in order to maintain the germ-free testing division during illness of other technical personnel.

Our activities in serology were: (1) the further study of the Universal Serologic Reaction, and (2) the continuation of the survey for "natural" hemagglutinins and "natural" anti-bacterial antibodies in the serum of germ-free and conventional animals. We hope to be able to make a detailed report in the next progress report.

V. PHYSIOLOGY AND PATHOLOGY

(Compiled by Dr. H. A. Gordon, Chief Physiologist, with the assistance of Wm. Scruggs and Patricia Wolfe.)

During the period covered by the present report, this laboratory made progress along three lines:

A. In respect to the basic germ-free program, it initiated a study dealing with the physiology of the Caesarian-born baby rat. This work has been undertaken to strengthen our knowledge about the earliest period of extra-uterine life which, so far, has been investigated only sporadically, and which plays an important role in some phases of germ-free research.

B. Next, this laboratory accumulated and evaluated data for the project concerned with the antibiotic effect in germ-free animals.

C. Finally, it carried on with the irradiation of germ-free and conventional rats in accordance with LOBUND's commitments to the AEC.

In addition, the laboratory performed as LOBUND 's pathology service unit. With the sharp increase in animal production, there was a concomitant increase in this activity during the past six months. The number of autopsies performed sums up as follows: chickens, 118; rats, 534; turkeys, 60; guinea pigs, 5; rabbits, 7; a total of 724 animals. This number is more than three times last semester's total.

The progress made with other older projects, not mentioned in the introduction, will be described in the final section of this report.

A. Physiology of the Caesarian-Born Baby Rat. Absorption of fat during the first hours of life in animals fed natural and artificial diets. A preliminary report.

1. Outline of the experiment.

The initiation and maintenance of normal absorption of food in Caesarian-born, handfed baby rats, kept on artificial diets is of

crucial importance in the development of germ-free animal colonies. The little understood physiological phenomena occurring in the intestine during the first few hours seem to play an important role in the life of the animal. Our purpose, therefore, is to study the absorption of various nutrients during this period as influenced by various agents (environmental and physiological, for example) which may affect absorption. The first food element studied in this series was fat.

2. Materials and Methods

(a) Experimental Animals

The infant rats were delivered by Caesarian section from time pregnancy dams (of either LOBUND or Sprague-Dawley stock) at term. They were then cleaned and kept at 30° - 34° C until they were ready to be fed. In each experiment the litters were thoroughly pooled so as to rule out possible litter differences.

The animals were sacrificed by decapitation, allowing the blood to flow into a centrifuge tube. From three to eight animals were used for each run of each aspect being tested. The blood of all animals of any given group was then pooled at the time of decapitation in order to cancel out as nearly as possible any individual variations.

(b) Variables tested.

Diets. Eighteen or 24% steam-sterilized cream (numbers represent the percentage of butterfat) prepared according to standard LOBUND formulas was used. The diets were given in the amount of 0.2 ml/rat.

Pre-treatment. Using the working hypothesis that in the course of natural parturition and immediately afterwards the alimentary canal of the new-born mammal receives some impetus which initiates proper absorption, we investigated the effect of the following substances by feeding them to the new-born rat: Tyrode's solution (in text referred to as Tyrode wash), amniotic fluid (Amfetin, Lilly) and the colostrum factor (reconstituted, homogenized chyle from the stomachs of 1 and 2 days old normal suckling rats). These substances were given per os. in the amount of 0.2 ml/rat.

Environmental changes. The animals were exposed to low (5° to 10°C) and high (39° to 41° C) temperatures throughout the period of observation.

Physiological effects. Tyrode's solution saturated with intestinal phosphatase (Armour Laboratories) of bovine origin was added to the 24% cream in a 1:3 proportion. The effect of phosphatase on fat absorption was studied in untreated and Tyrode-pretreated baby rats.

Pharmacological effects. Fat-absorption was studied in rats which were under parasympathetic stimulation (Prostigmin,* 5 μgm/rat s.c.) and depression (Atropine, 1 μg./rat s.c.). Starved and mother-fed animals were added as controls to the above mentioned groups.

(c) Methods and Schedule of treatment

All experiments were run in the open conventional environment.

*Kindly provided by Dr. M. J. Schiffrin and Mr. M. J. Stromberg of Hoffman-LaRoche, Inc.

One hour after delivery, the rats of each particular group were given the substances mentioned in the paragraphs "pre-treatment" or "pharmacological effects" (when so scheduled; otherwise, the animals received nothing at this time). Two hours following this, they received a single dose of the diet under test. Substances given per os. were introduced by way of stomach tube; subcutaneous injections were made under the skin of the dorsum. Two hours after the feeding, the animals of each group were decapitated and bled. Thus, the experiment terminated 5 hours after the delivery.

(d) Study of fat absorption

The absorption of fat was studied by the usual chylomicron (CHC) analysis of the systemic lipemia which occurs following the ingestion of fats. Counts were made in the serum obtained from the mixed arterial - venous blood resulting from the decapitation. This technique was supplemented by the histological study of fat particles in the epithelium of intestinal villi (results of the latter approach are not included in this report).

3. Results.

The preliminary results of this series are given in Table I.

TABLE I

	n	Chylomicrons		
		M	min	max
NPT Starved	30	1	0	3
NPT mother suckled	5	33	27	41
NPT 18% cream	15	22	10	34
Tyrode wash 18% cream	20	47	17	76
Tyrode wash 24% cream	15	99	81	111
Colostrum factor 18% cream	10	17	6	31
Amnion factor 18% cream	5	14	13	16
Cold 18% cream	15	2	0	6
Heat 18% cream	5	31	23	38
NPT phosphatase + 18% cream	30	57	39	81
Tyrode wash phosphatase + 18% cream	15	120	78	166
Saline s.c. 18% cream	10	47	21	42
Prostigmin 18% cream	20	13	0	31
Atropine 18% cream	10	54	43	71

Legend to Table I on preceding page as follows:

NPT = no pre-treatment
Cold, heat = as specified under "Materials and Methods"
n = number of chylomicron counts made
M = mean number of chylomicrons per standard field of vision
Min-max = minimum - maximum number of chylomicrons observed in standard field of vision.

4. Comment and Summary

As it is evident from the results of this series, the lipemia following the absorption of fat in baby rats can be considerably influenced by a number of environmental, physiological and pharmacological factors.

In the fasted new-born baby rat, the chylomicron count was practically nil.

The normal count in the dam-suckled animal was around 30/standard field of vision in our experiment.

If 18% cream is substituted for dams' milk, the CHC is slightly reduced. However, if the animals were previously fed some Tyrode's solution (Tyrode wash), the CHC was restored to normal in the group fed 18% cream, or even surpassed the normal in the 24% cream group.

The hypothetical "colostrum" and Amniotic fluid" decreased rather than increased the CHC when the animals were fed 18% cream.

Cold, as applied in this experiment, drastically reduced while heat had essentially no effect on the CHC.

Phosphatase added to the diet substantially increased the CHC. When this effect was combined with the previous administration of Tyrode, the CHC was increased nearly four-fold when compared with that of the normal, mother-suckled groups.

Prostigmin, a parasympathomimetic drug, often reduced considerably the CHC; probably through its effect on intestinal motility. Atropine seemed to cause a slight increase in the CHC.

Some conclusions of this experiment may be directly applicable to the problems of handfeeding baby rats.

B. Morphological effects of antibiotic feeding in germ-free and conventional chickens.

Details of this work have been reported at the Colloquium held at LOBUND Institute on June 4, 1952. All data presented have been reported earlier to ONR Washington.

C. A study of total body x-irradiation in germ-free and conventional rats.

1. An effort to determine the LD 50/30 days of total body x-irradiation in germ-free rats.

Methods

During the period covered by this report, 14 male germ-free and 13 conventional rats (as controls) have been exposed to total body x-irradiation. The germ-free animals were 4th generation germ-free and had been reared from weaning on our sterilized Diet L-128. The conventional animals were reared on sterilized Diet L-128 from weaning and were the progeny of parents which also had been reared on the same diet.

The animals were irradiated in two groups (for particular dates see Table II) of germ-free rats and their conventional controls; in both cases, however, the conventional animals were irradiated on the same day. Table II shows the survival time of these animals while Figure I illustrates these results graphically.

The physical factors of the irradiation were; 250 KVP, 15 ma., 15r/min., TSD 50 cm., HVL .25mm. Cu + .4mm Sn + 1mm. Al. The rats were restrained individually within aluminum annular cylinders of 1 mm. thickness. The irradiating beam penetrated a 1 mm. Al port cover before it reached the restraint. The total filter used, therefore, was .25 mm. Cu + .4mm. Sn + 3mm Al.

While the number of animals irradiated so far is insufficient to fix precisely the LD 50/30 days, our data indicate it to be greater than 600 and less than 800 roentgens for germ-free animals and lower than 600 roentgens for conventional LOBUND strain animals reared on the same diet as that of the axenized animals. It must be pointed out that our present results, when compared with the results reported in our last semi-annual progress report* seem to indicate that the LD 50/30 days is variable and is likely to be dependent on seasonal factors which are in operation.

In spite of the apparent seasonal variability of the LD 50/30 days, our data show that for doses of total body x-irradiation which are uniformly lethal within 30 days (800r and 1000r), germ-free rats survive approximately twice as long as their conventional controls. While the first germ-free animals were first generation germ-free and the later ones 4th generation germ-free, we do not feel that the differences in survival are due to this factor inasmuch as the conventional animals show a similar decrease in survival.

Clinical Courses

All the rats, both germ-free and conventional, which received 600r or more and which died within 30 days showed corneal opacities. The degree of opaqueness could not be correlated with irradiation dose. During the first 48 hours following irradiation all the animals showed normal behavior and took food and water which was offered ad libitum.

On about the third post-irradiation day most of the conventional animals showed anorexia and fecal and urinary incontinence, while in the germ-free animals these conditions were seen as terminal only. The conventional animals which survived the acute phase of the syndrome began

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*LOBUND-ONR Semi-Annual Progress Report, Contract N6-ori-83, Task Order III, 1 July 1951 - 31 December 1951.

TABLE II

The Effect of Total Body X-Irradiation on Survival Time of Germ-Free Rats

Germ-Free				Conventional Controls			
Number	Date Exposed	Dose (Roentgens)	Survival Days	Number	Date Exposed	Dose (Roentgens)	Survival Days
1033	4/16/52	1000	10	1035	4/16/52	1000	6
1034	4/16/52	1000	9	1036	4/16/52	1000	4
817	1/10/52	800	10	819	1/10/52	800	7
818	1/10/52	800	12	820	1/10/52	800	6
833	4/16/52	800	14	837	4/16/52	800	4
834	4/16/52	800	12	838	4/16/52	800	8
835	4/16/52	800	10	839	4/16/52	800	7
836	4/16/52	800	11	840	4/16/52	800	4
617	1/10/52	600	62+	619	1/10/52	600	18
618	1/10/52	600	17	635	4/16/52	600	11
633	4/16/52	600	62+	636	4/16/52	600	13
634	4/16/52	600	16				
333	4/16/52	300	17	335	4/16/52	300	15
334	4/16/52	300	62+	336	4/16/52	300	62+

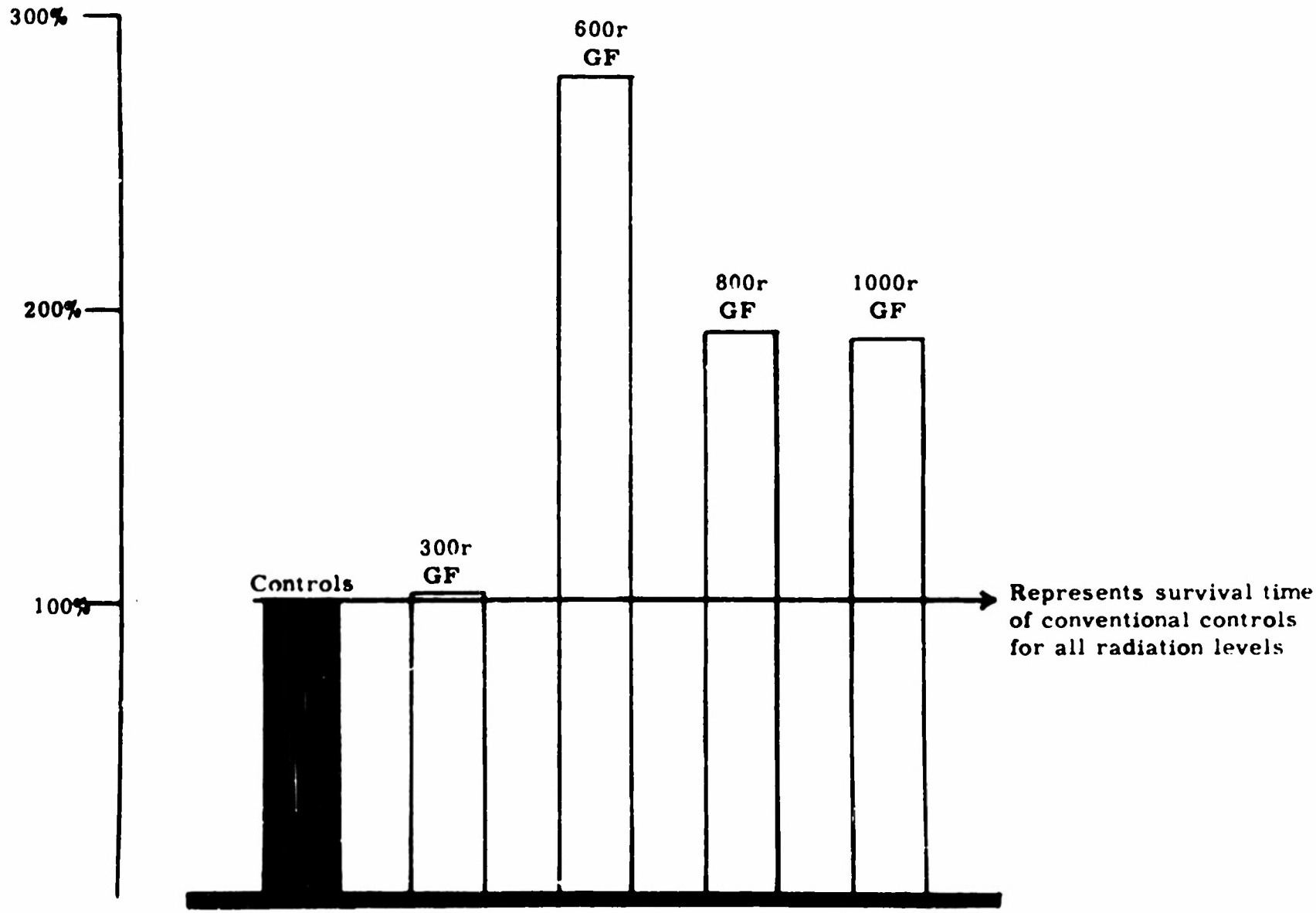
Average Survival Times

1000R = 9.5 Days
800R = 11.5 Days
600R = 39.25 Days
300R = 39.5 Days

Average Survival Times

1000R = 5 Days
800R = 6 Days
600R = 14 Days
300R = 38.5 Days

Figure I



When the average surviving time of the conventional control rats for each radiation-dose group is taken as 100%, the white bars show the relative surviving time of similarly irradiated germ-free rats.

to improve in these regards at about 6 days post-irradiation. Those conventional animals which did not survive the 30 day period invariably were moribund from 6 to 24 hours before dying; on the other hand, the germ-free animals usually died relatively abruptly and without showing signs of weakness. Occasionally a germ-free animal would become somnolent immediately prior to death, but this was the only visible indication that the animal was not normal.

Summary of Autopsies

Both groups of animals had certain macroscopic lesions which were common. These included decidedly brownish-yellow livers, hyperemic pulmonary changes, and hemorrhagic mesenteries. The lesions were similar in degree and kind for both groups but it must be emphasized that the germ-free rats showed these changes in approximately twice the time that the conventional rats showed identical lesions.

2. An effort to determine the effect of total body irradiation in dicontaminated rats.

Two dicontaminated, previously germ-free rats and four conventional rats were exposed to 2000r total body x-irradiation under the same physical conditions as those used with the germ-free rats. There was no difference in the survival time of the dicontaminated and in that of the conventional rats. The contaminants in the cage were micrococcus ureae and micrococcus (species as yet unidentified). The bacteriologist's report on the results of blood culture taken from a moribund dicontaminated rat is submitted below.

Bacteriology Report

Rat #2000 - 2 ♀(151 grams) which had received 2000r X-ray irradiation was sacrificed by exsanguination (heart puncture) on February 5, 1952. One ml heart blood, taken aseptically in a sterile syringe was inoculated into 10 ml fluid thioglycollate medium and incubated at 37°C.

The culture was examined 1 day, 3 days, 7 days, 20 days, and 48 days after inoculation but no evidence of bacterial growth was detected.

Six dicontaminated (with two micrococci) rats and six conventional rats were exposed to 800r total body irradiation, the conventional rats being irradiated 2 days prior to the exposure of the previously germ-free animals. One of the six previously germ-free rats was dead and in complete rigor at the time it was removed from the irradiation restraint. The results of this experiment are summarized in Table III.

3. Summary of the project

Fourteen germ-free and 13 conventional rats were exposed to total body x-irradiation in doses ranging from 300r to 1000r during the second quarter of 1952. While an insufficient number of animals have been used to pinpoint the LD 50/30 days it appears at this time that for germ-free rats, this dose lies between 600r and 800r, being rather close to 600 roentgens. Conventional rats irradiated on the same given day as the germ-free rats appear to have an LD 50/30 days, considerably lower than 600r yet greater than 300 roentgens.

TABLE III

The Effect of 800R Total Body X-Irradiation on Dicontaminated, Previously Germ-Free Rats

Previously Germ-Free			Conventional Controls		
Number	Date Exposed	Survival Days	Number	Date Exposed	Survival Days
847	5/7/52	2	840	5/5/52	5
848	5/7/52	12	841	5/5/52	8
849	5/7/52	14	842	5/5/52	8
850	5/7/52	14	843	5/5/52	10
851	5/7/52	29	844	5/5/52	10
852	5/7/52	< .1*	845	5/5/52	14

Average Survival Days	Average Survival Days
14 (Excluding #852) (11.8 Day including #852)	9

* Rat found dead upon removal from restraint

That germ-free animals at a given uniformly lethal dose tend to live twice as long as conventional animals is confirmed, but the total survival time for both categories of animals is one-half that of animals irradiated during the 3rd and 4th quarters of 1951. One possible reason for this variation in survival time is discussed.

The survival time of previously germ-free dicontaminated rats, while longer than that of conventional animals, is not as long as that of germ-free animals. This difference is taken to be a reflection of the previously germ-free and present bacterial status of these animals. A previously germ-free existence does not seem to affect the survival time of animals exposed to extremely high doses of x-irradiation (2000 roentgens).

D. Other projects

Among the other active projects, considerable progress has been made in the preparation of LOBUND Reports No. 3. In the tumor project we have evaluated the lymphocytic status of several conventional, methylcholanthrene injected, tumor bearing, and tumor-non-bearing chickens.

VI. VIROLOGY

(Compiled by John F. Reback and James A. Reyniers with the assistance of Miriam Sacksteder)

A. Introduction

In a recent discussion of purification procedures and criteria of purity with respect to viruses, Stanley and Lauffer* express the following credo:

> "It should be emphasized . . . . that as many tests as possible involving chemical, as well as physical methods of fractionation should be made, for a single negative result indicates only that the material is homogeneous under a given set of conditions. However, when attempts to fractionate the material by a variety of methods yield no evidence for the existence of an impurity, the burden of proof for the existence of an impurity can be regarded as resting upon those who wish to postulate its presence."

A somewhat analogous point of view may be taken as regards the question of virus-freeness of the Reyniers germ-free animal. From previous reports in this series, as from the present one, it should be evident that we are following several lines of investigation in the virus field, some bearing directly on the basic problem, some less so. All of our work, however, is conducted with a certain vigilance towards what the separate results might contribute, directly or indirectly, in answer to the question of the presence or absence of viral agents (active, latent, or symbiotic) in these animals. The demonstration of the presence of a symbiotic, inseparable virus (hitherto inapparent) in the GF host would of course conclude this matter at that point. However, if in the course of time no positive demonstration appears to be forthcoming, then "proof" for the negative will be dependent on such bits of oblique or circumstantial evidence as may have been garnered, more or less incidentally, along the way. For the present, we may operate

*N.B. - Stanley, W. M. and Lauffer, M.A. "Chemical and Physical Procedures", Ch.2, p. 30, in "Viral and Rickettsial Infections of Man", ed. by T. M. Rivers, 1948. Lippincott, Philadelphia.

on the assumption that the standard bacteria-free animal is also virus-free, remaining watchful, meanwhile, for any sign that this may not be so.

B. Current Studies

Our observations on the action of the virus of Rous sarcoma on GF chicks have perhaps developed sufficiently to be presented in some detail, as is done in Section C of this report. It is felt, however, that brief mention should at least be made of certain other problems which have also taken our attention, in greater or lesser measure, during the past six months. These may be listed as follows:

1. Attempt at induction of lymphomatosis in GF chicks with filtrates of tumor material.

2. Evaluation of the GF chick as a possible host for the virus of infectious hepatitis (human). Note may be made at this time of the possible serial passage through 3 consecutive groups of GF chicks of a filtrable agent obtained initially from a pool of acute phase human hepatitis sera.

3. There appears to be no effect on inoculation (IC, IM, IV, and IP) of the virus of Theiler's mouse encephalomyelitis into GF chicks. However, tests for survival of the virus are being made by titration in mice of serum, brain, and intestinal contents from the inoculated GF chicks.

4. Attempt at X-ray stimulation (a) of the virus of Rous sarcoma and (b) of the filtrable agent from human infectious hepatitis in GF chicks (a) after subclinical response to inoculation and (b) after contact exposure.

5. Effect of transmission of serum from X-ray irradiated GF chicks to standard GF and standard conventional chicks.

6. Observations for, and cursory attempts at in vitro cultivation of, possible parasites (or symbionts) in the RBCs of normal GF and conventional chicks and other hosts (the "Rottino RBC parasite").

7. Attempts to induce propagation or survival of animal viruses in association with microbial cells. We are giving time principally to the viruses of Theiler's mouse encephalomyelitis, Rous chicken sarcoma, influenza PR8, and Kikuth's canary pox, with limited attention to the pneumonia virus of mice (PVM) and Baker's feline pneumonitis virus. The microbial cells being used in conjunction with these agents are Saccharomyces cerevisiae Carlsbergensis, Rhodotorula rubrum, a pure (British) baker's yeast, Staphylococcus aureus, Escheriohia coli, Bacillus subtilis, and Trypanosoma cruzi. Colorimetric and pH effects of the virus-microbe associations are followed, and tests for the viruses are made, after a series of transplants, by serologic methods or by appropriate inoculation into mice, chicks, or embryonated eggs. Some indicative results have been obtained, and some of the work is being repeated with some refinement and greater emphasis on control.

These investigations are underway. Detailed report will be made in each instance at the appropriate time.

C. Rous Sarcoma in GF Chicks

The remainder of this report is given to our observations on the effect of Rous sarcoma virus in 1 to 2 month old GF chickens. Some deviation from the usual pathogenesis as observed in the conventional bird is noted to occur with the inoculation of this agent into the GF chick; probably more noteworthy, however, is the similarity in overall response in the two types of host. We have already reported* the similarity in Newcastle virus pathogenesis under the two ecological states (GF and conventional). At least these two viruses (Rous and NDV) evidently are capable of eliciting the syndrome and pathology commonly attributed to them without the assistance of, or the need of any sort of synergism with, other microorganisms.

Materials and methods

The viral agent used throughout these experiments was obtained originally from the Stine Laboratory of the Dupont Company, through the courtesy of Dr. C. O. Prickett. Six half-grown Rous-infected chickens were received by us on January 27, 1951 and all of our work has been done with the agent isolated from these birds.

Several slightly different methods were used in the preparation of the required virus ultrafiltrates. Conventionally, freshly harvested Rous tumor tissue was sliced into small pieces and these were minced with saline, in a TenBroeck apparatus, to yield a suspension containing approximately 30 percent of solids (V/V). The value of further maceration with sand, or of antolysis, or of a combination of these two procedures, to liberate the virus elements for purpose of ultrafiltration, was found to be equivocal, as determined by the activity of the resultant ultrafiltrates (Table 1). In some instances, then, the TenBroeck homogenate was further ground in a mortar together with sterile Berkshire sand, the whole thereafter centrifuged a short time at low speed in order to sediment the sand. When resort was made to autolysis this was done by holding the saline - suspended 30 percent macerate overnight at 4 C.

Filtration of the suspension was then made through a coarse (C) fritted-glass funnel (40 micron pore size). Finally, filtration of the C filtrate was made through ultrafine (UF) fritted glass (1 to 2 micron pore size). In the latter process, the bulk of the C filtrate was held at 4 C, and aliquots of this were transferred to the UF filter funnel at appropriate intervals; while the ultrafiltrate was held at 0 C throughout the filtration period. Thus active filtrates were obtained in 7 of a total of 9 separate trials (Table 1). Analysis of the results fails to disclose an optimal method. Intramuscular inoculation of 0.25 ml (and possibly less) of such filtrates will produce tumor in 30-day chicks.

The ultrafiltrates were ampuled aseptically, final portions being tested for bacterial sterility in thioglycollate, nutrient, and Sabourand's broths and in nutrient and Sabourand's agar media. If not immediately used, the sealed filtrate was quick-frozen and stored at -20 to -60 C. As need arose, the ampule was cleaned with sulfuric acid - dichromate solution, rinsed with distilled water, immersed for a time in 2 percent mercuric chloride solution, and then passed into the GF cage via the germicide trap.

*N.B. - LOBUND Institute -ONR Semi-Annual Report, 31 January, 1952

TABLE 1 – PREPARATION AND ACTIVITY OF ROUS VIRUS ULTRAFILTRATES.

Filtrate No.	Bact. Test	Tissue conc. of Ten Broeck Homogenate (percent solids, W/V)	Treatment of Ten Broeck Homogenate	Inocula (range in ml)	Tumor Incidence/Inoculations	
					Conventional Chicks	GF chicks
I	Sterile	50	None.	0.5	3/3	—
II	Sterile	50	Ground & sand.	1.0	3/3	—
III	Sterile	30	None.	0.25 to 0.5	2/3	0/2
IV	Sterile	33	None.	0.8 to 1.0	1/1	1/1
V	Sterile	33	Ground & sand, then autolyzed.	0.6 to 2.0	0/3	0/2
VI	Sterile	18	Autolyzed, then ground & sand.	0.5 to 1.0	3/3	1/2
VII	Sterile	30	Autolyzed, then ground & sand.	0.5 to 0.75	2/2	1/2
VIII	Sterile	30	Autolyzed.	0.7	0/3	—
IX	Sterile	25	Ground & sand.	0.5	3/3	3/4

Results of Experiment #1

Attempt to establish Rous sarcoma in two 30-day GF White Leghorn chicks was without success. Contamination of the GF cage occurred about 13 days after inoculation of the chicks. These birds, removed from the cage at the time of contamination, failed to show tumor, when held under conventional conditions for a period of nearly 3 months. That this ultrafiltrate (#III) contained active Rous sarcoma elements was demonstrated by the results in 2 of 3 conventional control chicks, inoculated at the same time as the (initially) GF chicks and showing tumor in about 2½ weeks. However, the third bird of the control group also showed no sign of "take" as long as 3 months after inoculation.

Results of Experiment #2

Of two ultrafiltrates, one (#IV) was found to be active in both GF and conventional 37-day chicks, while the second (#V) proved ineffectual for either ecological state (Table 2). Both tissue homogenates, from which the corresponding filtrates were prepared, were active in conventional birds (Table 2).

The same ultrafiltrate (#IV) induced externally detectable tumor in a GF bird (#172, Table 2) 3 days earlier than in a conventional bird (#F4, Table 2), though the affect on the latter proved more devastating in terms both of extent of sarcoma and of survival time. Conventional chicken #F4 died on the 10th day following appearance of tumor. This bird showed the marked green discoloration of the ventual surface, usually associated with Rous tumor in such conventional birds. Autopsy disclosed a typical massive sarcoma in the tissues of the right breast (area of inoculation), and gross and microscopic metastases were found in the liver. All other organs and tissue appeared normal.

Eleven days after appearance of tumor in GF bird #172, contamination of the GF unit occurred. Still normally active on the 13th day, this bird, now 2 days contaminated, was taken for sacrifice and comparison with the conventional control (#F4). Chicken #172 showed a marked but delimited tumor in the tissues of the right breast (area of inoculation). There was no discoloration. No metastasis could be found. It was grossly evident that the tumor was in a state of regression during the period just prior to contamination and sacrifice of the chicken. Moreover, microscopic examination revealed a high lymphocyte response throughout the area of the tumor, suggesting that the host organism may have had the situation under control at the time of autopsy.

It is surmised that the tumor would have disappeared completely had the chicken been spared. Though rare, such complete regression has been observed in conventional birds in this laboratory (cf. Experiment #3) and elsewhere*.

The green discoloration invariably found in the region of the breast tumor in conventional birds, whether inoculated with filtrate or cell hash, was absent in the filtrate-inoculated GF bird. Bacteria have not thus far been cultivated from this green fluid (conventional birds). It is suggested that the material may be a decomposition product of heme structures in the blood.

* N.B. -- Personal communication from Dr. C .O. Prickett.

TABLE 2 CHRONOLOGICAL SUMMARY OF EXPERIMENT 471L2 (GF-ROUS - #2).

CHICK No.	ECOLOGICAL STATE	INOCULUM PREP.	INOCULUM NATURE	INOCULUM ml.	MAY 31	JUNE 6	11	15	19	20	21	22	23	24	25	26	27	28	29	30	JULY 1	2	3	4	5	6
TEST:																										
172 (♂)	GF	IV	FILTRATE	1.0		+	0	0	?	–	–	T	T	T	T	T	T	T	T	T	T	T	T	T	X_S	
175 (♀)	GF	V	FILTRATE	1.2		+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
176 (♀)	GF	V	FILTRATE	2.0		+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
CONTROLS:																										
F4	CONTROL	IV	FILTRATE	0.8		+	0	0	0	0	–	?	–	–	T	T	T	T	T	T	T	T	T	T	X	
F5	CONTROL	V	FILTRATE	0.6		+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
F6	CONTROL	V	FILTRATE	1.0		+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
F7	CONTROL	V	FILTRATE	0.9		+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
M8	CONTROL	IV	CELL HAM	0.9		+	0	?	–	T	T	T	T	T	T	T	T	T	X_S							
M9	CONTROL	IV	CELL HAM	1.0		+	0	?	–	T	T	X														
M10	CONTROL	V	CELL HAM	1.0	+	0	–	T	X																	
M11	CONTROL	V	CELL HAM	1.0		+	0	?	–	T	T	T	T	T	T	T	T	X_S								
M12	CONTROL	V	CELL HAM	1.0		+	0	?	–	T	T	T	T	T	T	T	X									
M13	CONTROL	V	CELL HAM	1.0	+	0	–	T	T	X																
UNINOC:																										
P14	CONTROL	—	—	—		–	0	0	0	0	0	0	0	0	0	0	0	X_S								
P15	CONTROL	—	—	—		–	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
P16	CONTROL	—	—	—		–	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
P17	CONTROL	—	—	—		–	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
CULTURES TAKEN IN GF UNIT					S	S	S								S							S	C			

DISCONTINUED

KEY: + INOCULATED. — NOT EXAMINED. S CAGE STERILE. C CONTAMINATED. ? RESPONSE IN DOUBT. 0 NO TUMOR, T TUMOR. X DEATH FROM TUMOR. X_S SACRIFICED ☠ TUMOR.

Results of Experiment #3

Two cell-containing coarse (C) filtrates and their corresponding cell-free ultrafine (UF) filtrates were inoculated, the former into conventional chicks alone, the latter into both conventional and GF chicks. Table 3 is a chronological record of tumor incidence and mortality. It may be noted that, in general, the C filtrates induced recognizable tumor (usually more than merely palpable) within 2 weeks after inoculation (conventional chickens), with death resulting in 1 to 2 weeks following the initial appearance of tumor.

A 1:2 incidence was obtained with each of the two ultrafiltrates in both 33-day and 60-day White Leghorn chickens held under otherwise GF conditions (Table 3). Aliquots of the same ultrafiltrates were active in conventional birds. A saline homogenate prepared from the induced proliferative growth in one of the GF birds (#661) was shown to be capable of causing the typical sarcoma in conventional chickens.

Of two 33-day GF birds inoculated intramuscularly with Ultrafiltrate VI, one (#651) gave no response over a period of 73 days. At that time, this bird, still germ-free, was exposed to about 430r of X-rays. After another month this chicken was sacrificed and examined. No Rous pathology was found. The second GF chick (#656) began to show palpable growth on about the 16th day. In 2 days this developed into what appeared, to external examination, to be a fullblown tumor. The chicken died on the 19th day. Examination disclosed a tumorous mass with very considerable cholequation. No similar effect due to Rous virus inoculation has been observed in this laboratory. The considerable liquefaction (to be distinguished from the usual hemorrhagic necrotic center) would point to an atypical pathogenesis. Breast tissue was replaced by a viscous gelatin-like material. There was no indication of metastasis to the viscera. Though some possible anemia was noted, the organs otherwise appeared normal.

One month later, Ultrafiltrate VII was introduced into the same GF unit. A second group of two 60-day birds was inoculated. Again a 1/2 incidence obtained. Chicken #654 was held under observation for 77 days with negative results. Chicken #661, on the other hand, exhibited tumor in about 3-1/2 weeks. Initially, under palpation, the tumor seemed atypically soft. In the ensuing 2 weeks, this developed into a rather typical massive sarcoma (external inspection). During the first week moderate discoloration was observed, suggesting the green discoloration usual with Rous tumors in conventional birds (cf. Experiment #2). By the end of the second week, however, no green discoloration was apparent, and the external appearance of the tumor was that of a pasty yellow-white mass. The bird became sluggish, then sedentary, then moribund. Death, attributable to the tumor, occurred 42 days after inoculation. Germ-free conditions had been preserved throughout. Autopsy revealed a typical Rous sarcoma extending throughout the entire right breast. A "walling off" or histologic delimitation to this region, however, was apparent. There was no bone degeneration or destruction. The liver was slightly engorged but otherwise normal. Hemorrhagic areas from mesenteric vessels were found in the region of the gizzard and elsewhere. The spleen, though somewhat friable, otherwise appeared normal. Lungs, kidneys, gonads, heart, intestines, etc., appeared normal.

It may be noted (Table 3) that under GF conditions a shorter incubation period obtained, with production of rather marked off tumor growth showing no free green fluid or tissue discoloration.

Contamination of the GF unit with Bacillus subtilis was detected 99 days after the first inoculations with Rous virus, and 30 days after the last tumor-bearing bird in this cage had died.

(X-ray exposure of a virus-inoculated but resistant bird failed to induce tumor formation within a 30-day period. Also one instance of complete regression could not be re-activated by irradiation. Nor did X-ray treatment produce any proliferative response in a bird previously inoculated with normal chicken tissue and maintained in close contact with tumor-bearing birds for two months prior to exposure. See Table 3.)

Results of Experiment #4

Incidence of tumor was noted in 3 of 4 filtrate-inoculated 28-day White Leghorn chicks while these were maintained under otherwise GF conditions; thereafter, however, fungus and bacterial contamination of the GF unit occurred, so that mortality, attributable to tumor growth, in the case of these 3 birds, was observed only under contaminated conditions (Teble 4). Chronological tabulation is made of the incidence and development of tumor in these GF birds and in conventional controls, in consequence of the intramuscular inoculation of both groups with the same virus-containing ultrafiltrate (# IX). Included are the results with a homogenate of the proliferative tissue from one of the "GF" birds (#383), prepared just after the GF unit became contaminated; this homogenate was shown to be capable of inducing Rous sarcoma in 2 strains of chicks held under conventional conditions. Note may be taken (Table 4) of the two attempts at X-ray stimulation towards the end of the experiment.

As has been the practice in this report, only the pathological findings with the GF chicks will be detailed:

GF #383 — 28-day germ-free bird inoculated intramuscularly with 0.5 ml of Ultrafiltrate IX on April 22nd. Incidence of tumor detected 13 days after inoculation, though tumor did not develop into an unmistakable form until about 3 weeks after inoculation. Incidence of tumor was characterized by a definite pinkening and a small upraised area over the site of inoculation. After 2 days the pinkish elevated area measured ca. 0.5 x 1.0 cm. In another 4 days an unquestionable tumor had developed. There was no discoloration of the area. The tumor was allowed to grow until May 28th, at which time sacrifice of host was made. The GF state had been maintained to within a day or two of sacrifice, at which time a mold contamination occurred. Examination showed a massive sarcomatous growth with no discoloration of the tissue and no free green fluid. Five or six separate secondary foci, yellowish and caseous-like in character, though rather firm, were found in the tumor tissue of the right breast. There were numerous metastases to the liver, spleen, gonads, and the blood vessels of the mesentery. The "potato eyes" effect on the spleen was especially striking. Lungs and intestines were normal; there was no metastesis to the left breast.

TABLE 3 CHRONOLOGICAL SUMMARY OF EXPERIMENT NO. 471L2 (GF-ROUS-#3).

CHICK NO.	ECOLOGICAL STATE	INOCULUM PREP.	INOCULUM NATURE	INOCULUM ml.	SEPT. 18	SEPT. 25	OCT. 1	OCT. 2	OCT. 4	OCT. 5	OCT. 6	OCT. 7	OCT. 8	OCT. 10	OCT. 15	OCT. 16	OCT. 18	OCT. 23	OCT. 26	OCT. 30	NOV. 6	NOV. 8	NOV. 10	NOV. 13	NOV. 22	NOV. 23	NOV. 26	NOV. 30	DEC. 5	DEC. 12	DEC. 15	DEC. 21	DEC. 26	DEC. 31
TEST:																																	
651	GF	VI	UF	1.0	↓	0	0	0	0	0	?	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	e	0	0	0	0	0	X_S
656	GF	VI	UF	0.5	↓	0	0	0	?	?	T	D_c																					
654	GF	VII	UF	0.75	–	–	–	–	–	–	–	–	–	–	↓	0	0	0	0	0	0	0	0	0	0	0	0	0	?	0	0	0	0	X_S
661	GF	VII	UF	0.5	–	–	–	–	–	–	–	–	–	–	↓	0	0	0	0	0	0	–	T	T	T	T	D							
CONTROLS:																																	
III-1	CONVENTIONAL	VI	UF	1.0	↓	0	0	0	0	0	0	0	0	0	$D_?$																		
III-2	CONVENTIONAL	VI	UF	0.5	↓	0	0	0	–	T	T	T	T	T	T	T	D																
III-3	CONVENTIONAL	VI	UF	0.5	↓	0	0	0	0	0	T_A	T_A	T_A	T_A	T_A	T_A	–	0*	0	0	0	0	0	0	0	0	0	e	0	0	0	0	0	X_S
III-4	CONVENTIONAL	VI	C	1.0	↓	0	T	T	T	T	T	T	T	X_S																			
III-5	CONVENTIONAL	VI	C	0.5	↓	0	T	T	T	D																								
III-6	CONVENTIONAL	NORMAL TISSUE	C	1.0	↓	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	e	0	0	0	0	0	X_S
III-7	CONVENTIONAL	VII	UF	0.75	–	–	–	–	–	–	–	–	–	–	↓	0	0	0	0	0	0	0	0	0	–	T	T	T	X_S					
III-8	CONVENTIONAL	VII	UF	0.5	–	–	–	–	–	–	–	–	–	–	↓	0	0	0	0	0	0	–	?	T	T	T	T	T	T	D				
III-9	CONVENTIONAL	VII	C	0.75	–	–	–	–	–	–	–	–	–	–	–	↓	0	0?	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	X_S
III-10	CONVENTIONAL	VII	C	0.6	–	–	–	–	–	–	–	–	–	–	–	↓	C	0	T	T	T	D												
CULTURES TAKEN IN GF UNIT.					S	S		S		S		S		S	S		S	S	S	S		S	S	S	S		S		S	S		S	C	C

*N.B. - FIRST OBSERVED REGRESSION (IN THIS LABORATORY) IN CONVENTIONAL (I.P. CONTAMINATED) BIRDS.

KEY:

↓ INOCULATED.
– NOT EXAMINED.
e X-RAY IRRADIATED.
T TUMOR.
T_A ATYPICAL TUMOR.
? UNCERTAINTY.
D DEATH FROM TUMOR.
D_c DEATH, CONSEQUATED TUMOR.
$D_?$ CAUSE OF DEATH UNCERTAIN.
S CAGE STERILE.
C CONTAMINATED.
X_S CHICK SACRIFICED FOR EXAMINATION.

TABLE 4 CHRONOLOGICAL SUMMARY OF EXPERIMENT NO. 4712 (GF - ROUS - # 4).

CHICK NO.	ECOLOGICAL STATE	INOCULUM			APRIL		MAY											JUNE 1952							
		PREP.	NATURE	ml.	22	29	2	5	7	8	9	17	20	23	26	28	29	5	6	11	14	16	17	18	24
GF-TEST																									
381	GF	1X	UF	0.5	↓	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	e	0
382	GF	1X	UF	0.5	↓	0	0	0	0	0	0	T	T	T	T	T	T	T	T	T	D_X				
383	GF	1X	UF	0.5	↓	0	0	?	I	I	I	T	T	T	T	D_S									
390	GF	1X	UF	0.5	↓	0	0	0	0	0	?	I	I	I	I	I	I	T_A	T_A	T_A	T_A	T_A	T_A	D_S	
384	GF	—	—	—	—	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	e	0
CONTROLS																									
111	CONVENTIONAL	1X	UF	0.5	↓	0	0	—	—	0	0	0	0	0	0	0	0	0	0	0	—	—	—	—	T
116	CONVENTIONAL	1X	UF	0.75	↓	0	0	—	—	0	0	?	T	T	T.	T	T	T	D_S						
118	CONVENTIONAL	1X	C	0.5	↓	0	0	—	—	T	D_X														
DEM A	CONVENTIONAL	383T	HASH	0.5													↓	—	—	I	T	D_X			
DEM B	CONVENTIONAL	383T	HASH	0.5													↓	—	—	I	T	D_X			
521	CONVENTIONAL	383T	HASH	0.5													↓	—	—	I	I	T	D_X		
622	CONVENTIONAL	383T	HASH	0.5													↓	—	—	I	I	T	T	T	D_S
CULTURES TAKEN IN GF UNIT					S	S	S	S	S	S	S	S	S	S	S	C_m	C_m	C_m	C_m	C_m	C_{mc}	C_{mc}	C_{mc}	C_{mc}	—

KEY:

↓ INOCULATED.
— NOT EXAMINED.
0 NO EXTERNAL SIGN OF TUMOR.
e X-RAY EXPOSURE.
? DOUBTFUL SIGN.
I INCIPIENT TUMOR.
T TUMOR.
T_A ATYPICAL TUMOR.
D_X DEATH FROM TUMOR.
D_S SACRIFICED FOR EXAMINATION.
S CAGE STERILE.
C CONTAMINATED { C_m MOLD; C_{mc} MOLD, COCCI.

GF #382 — GF bird inoculated intramuscularly with 0.5 ml of Ultrafiltrate IX, at 28 days of age. Incipient tumor detected 23 days after inoculation. Area continued to enlarge to a marked growth during the ensuing 8 days. There was no discoloration. In the following days the tumor mass became pronounced but appeared to be circumscribed. The bird was allowed to go to death (June 14th) 53 days after inoculation, 30 days after the first signs of tumor incidence. A rather firm tumor was found, ca. 6 to 7 cm. in diameter with one caseous focus only at the site of inoculation. Some necrosis (not marked) with hemorrhage was found in the center of the sarcomatous mass. Spleen retarded. Gall bladder engorged. Bilious stools and considerable bile throughout the intestinal tract, but especially noticeable in the rectum. Possibly some involvement of the right kidney. Lungs, heart, liver, gizzard, gonads, left kidney not involved and normal in appearance. Sternum deformed, and tumor growth found progressing to a slight extent around the point of the keel bone to the left breast tissues. Tumor incidence occurred under GF conditions, but fungus and bacterial contamination developed about 2-1/2 weeks prior to death of this bird from tumor.

GF #390 — GF bird inoculated intramuscularly at 28 days with 0.5 ml of Ultrafiltrate IX. Tumor incidence was detected about 17 days after inoculation. The incipient tumor in this case was characterized by an area of hardening at the site of inoculation. The small indurated area was palpable for a period of 3-1/2 weeks before more definite signs of tumor appeared. About 44 days after inoculation a hard atypical delimited tumor growth became apparent. The growth developed somewhat during the ensuing 2 weeks, but remained circumscribed. The transition from the incipient to the "atypical" sarcomatous stage occurred only after the GF unit has become contaminated with a mold. The chicken was sacrificed on June 18th, 57 days after inoculation. This was perhaps the "cleanest looking" Rous-inoculated bird thus far posted by us. All tissues were firm, clean, normal. The tumor in the right breast was found to be an integrated ball of firm tissue, ca. 3 cm. in diameter, except for a small (ca. 0.5 cm) necrotic-hemorrhagic area in the upper central part of the tumor. The tumor "ball" seemed actually to "pull away" from the remainder of the muscle-connective tissue in the right breast region. There was no green discoloration. Yellowish tissue was noted in certain centrally situated sections of the tumor. The general appearance of the breast tumor, as dissected out from under the skin, was reminiscent of an unlaid hen's egg; section of this, however, disclosed the firm sarcomatous tissue already described. Liver, lungs, heart, spleen, gonads, kidneys, intestines, gizzard, etc., appeared normal. The left breast tissue was not affected. No sign of metastasis from the right breast was observed.

GF #381 — GF chick inoculated intramuscularly with 0.5 ml of Ultrafiltrate IX on April 22 at 28 days of age. Contamination of the GF unit with a mold was detected on May 28 and with a coccus on June 14th. No external signs of tumor have become manifest in the 12 weeks since this chicken was inoculated. On June 18th, an attempt at stimulation of the virus was made by exposure of the host to 300r of X-rays. No effect whatsoever has been observed in the 4 weeks since irradiation.

GF #384 — Uninoculated GF chick maintained in close contact with Rous-inoculated birds, from 28 days of age. No contact transmission observed during 12-week period, despite close association with tumor-bearing birds. Bird exposed to 300r of X-rays on June 18th. No effects after 4 weeks.

Discussion:

It is clear from these experiments that ultrafiltrates of Rous tumor tissue will induce the sarcoma in GF chicks. Close comparison at this time of the effects of this virus under the two ecological states (GF and conventional) is made difficult (1) because of the limited number of observations to date under strictly GF conditions, and (2) because even under conventional (natural) conditions the virus of Rous sarcoma appears to manifest a rather variable pathogenesis.

However, a few statements, subject of course to possible future revision, may be made. The pathogenesis of Rous virus in conventional chickens and in the limited number of GF chickens thus far studied would seem to differ in the following points.

Effect of Rous ultrafiltrate inoculated intramuscularly into conventional chickens	Effect of Rous ultrafiltrate inoculated intramuscularly into GF chickens
1. Average incubation period of 3 to 4 weeks.	1. Average incubation period of 2 to 3 weeks.
2. Discoloration of tumor area due to dark green fluid.	2. No discoloration of tumor tissue.
3. Progressive tumor growth without sharp boundaries	3. "Walling off" or delimitation of tumor growth
4. Usually marked hemorrhagic necrosis in center of tumor mass.	4. Limited necrotic hemorrhagic center, if present at all.

The GF pattern here presented is based on observation of chickens #172 (Experiment #2) #656 and 661 (Experiment #3), and #382, 383, and 390 (Experiment #4), all of which developed tumor under otherwise GF conditions; however, in Experiments #2 and 4, the GF units became contaminated prior to the time of death or sacrifice of the tumor-bearing birds.

It seems that a shorter incubation period, compared to that observed under conventional conditions, may be associated with the otherwise GF state of the host. Otherwise, no great difference in susceptibility of GF as compared with conventional chickens has been observed. Ultrafiltrates active in one ecological state have been found, with one exception (#III, Exp. #1) also to be active in the other.

Under conventional conditions, a thick dark green fluid is usually found ventral to the breast tumor, in the boundary between the epidermal and muscle layers. In later stages, portions of the proliferative tissue itself may show discoloration. The source of the free green fluid has not been definitely determined. However, extensive central necrosis with hemorrhage usually occurs in the breast tumors of the filtrate-inoculated conventional control chicks; whereas such necrotic centers either do not appear or are not as marked in the tumors of the virus-inoculated otherwise GF birds. This may explain the absence of the green discoloration (heme compounds?) in the sarcomatous tissue of the latter. It has not been possible to cultivate bacteria from the green fluid.

Again because of the few observations made so far where the GF state was preserved from inoculation of virus to death of host, it is not possible to make any statement, desirable as it might be, as regards comparable life duration under the two ecological states (GF and conventional), following incidence of tumor. Certain other factors also argue against any interpretation of the data in terms of differences in life duration of tumor-bearing birds under GF as compared with conventional conditions: (1) moribund birds often were sacrificed for purposes of pathological examination, and it is not possible to say how much longer a "moribund" bird might have lived; (2) precise date of onset of tumor was sometimes difficult to determine; (3) filtrate dosages administered were not always strictly equivalent.

Other than in the differences suggested in the preceding paragraphs, it would appear (though this observation may prove premature) that pathogenesis under GF conditions, due to Rous virus, parallels the picture found in the inoculated conventional birds. This would seem to hold as regards duration and extent of tumor growth at the site of inoculation, degree and variability of metastasis, rate of tumor incidence/filtrate inoculations made, possibility of regression of tumor, inevitable mortality attributable to the proliferation, and so on. However, the possible differences thus far observed may be important enough to merit further study.

D. Summary

Description is given of our experiments with the virus of Rous sarcoma in GF chicks. A provisional discussion is offered covering some of the similarities and differences in Rous virus pathogenesis under the two ecological states (GF and conventional). The similarity in pathogenesis in the two types of host would appear more noteworthy than the total of the observed differences. However, strictly on the basis of the limited number of observations here reported, attention should perhaps be drawn to the following possible "deviations" noted with the GF birds: (1) a shorter incubation period before incidence of tumor (as detected by palpation and external inspection); (2) the absence of free blue-green fluid and tissue discoloration in the breast tumor; (3) a lesser hemorrhagic necrosis in the breast tumor tissues; and (4) the more definite histologic boundary of the tumor mass.

VII. SUMMARY

I. Administrative Section

The present personnel status and changes in physical plant are reported. Also reported are publications; a colloquium on antibiotics; and the collaborative programs.

II. Apparatus and Techniques Section

A. A report is submitted of the refinements in germ-free apparatus and methods. Also included is a summary of germ-free animal production during the past six months.

B. A report is given of preliminary steps being tested to change the method of entry for the diver in connection with the germ-free colony apparatus.

III. Biochemistry and Nutrition Section

The survey problems represent our biggest effort. In biochemistry the chicken survey is over the first hurdle - gross analysis - and is getting set for a more specialized type of work; the survey of the germ-free rat is in full swing. The nutrition survey of the germ-free chicken indicates few, if any, qualitative differences between the germ-free and conventional chicks, while the nutrition of the germ-free rat is a problem of the future.

Antibiotics were found to exhibit no positive effect action on the growth rate of germ-free chicks and turkey poults.

The dental caries project is into the second phase (that of inoculated rats) while the results of the first phase (showing caries do not occur in germ-free rats) are being written up.

Although germ-free rats (6) were found to have no liver necrosis in those experiments when most of the control conventional rats had died with the disease, this experiment is to be repeated to give a more conclusive answer.

The "LOBUND Merry-Go-Round" now revolves with a purpose - to test the effect of environment on variation in experimental work with chicks.

Germ-free turkeys were reared successfully.

IV. Bacteriology and Serology Section

The work in the bacteriological testing of germ-free apparatus has increased as more equipment for germ-free experimentation has become available. It has thus been necessary to divert considerable energy to this phase. A new helper was hired July 2, 1952 to assist in this work.

Spore-forming organisms seen in the feces of germ-free rats are discussed. These organisms have not been cultivated by routine culture technique, embryonated egg technique or by special procedures used for overcoming "dormancy" of endospores.

A list of 22 organisms found to be present in the isolated control rat colony is given. The list covers the period of operation from November 6, 1950 to February 6, 1952. On February 6, the colony was still apparently salmonella, worm and insect free but had acquired a protozoan, Chilomastix, from a wild mouse which had gained entrance to the animal room.

Twenty-three stock strains representing 14 bacterial genera were checked for their ability to grow in laboratory media to which 1% activated carbon was added as an absorbant. Such media has been proposed for sterility testing of antibiotics for which no inactivators are known. Poor growth of streptococci and lactobacilli in 1% carbon medium necessitates reduction in carbon concentration, probably to the 0.1% level.

Qualitative and quantitative studies are being conducted on the incidence of lactobacilli in the oral cavity of rats maintained mono-contaminated vs animals maintained in a conventional laboratory animal room environment. These studies will later be correlated to caries incidence in the various groups or individuals within the group.

The bacteriologic changes occurring in the cecal contents of chickens fed growth-promoting levels of antibiotics are reported. Although some changes were noted, none were considered responsible for the growth promotion effect.

V. Physiology and Pathology Section

A. In an effort to study the physiology of food absorption in Caesarian born rats, we have examined the absorption of fat in such rats during the first 5 hours of life. We found that the lipemia which follows the ingestion of fat can be greatly influenced by well defined environmental, dietary, physiological, and pharmacological agents.

B. Reference is made to the work of this laboratory on the growth effect of antibiotics in germ-free chicks. The data obtained was presented at a colloquium held 4 June 1952 at Notre Dame. The detailed proceedings of this colloquium have already been reported to CNR Washington.

C. It appears that the LD 50/30 days of total body x-irradiation lies between 600r and 800r for germ-free rats. Comparable conventional rats irradiated on the same day appear to have an LD 50/30 days considerably lower than 600r, yet greater than 300r. Germ-free animals at a given uniformly lethal dose tend to live twice as long as the conventional controls.

VI. Virology Section

A. A new "working hypothesis" is suggested as regards the problem of the virus-freeness of the Reyniers germ-free animal .

B. A briefly elaborated listing is made of problems currently under study by the Virus Laboratory.

C. Observations on the effect of the virus of Rous sarcoma in GF chicks are presented. The similarity in histologic response of the GF as compared with the conventional host is the more noteworthy feature; however, some observed differences are reported.